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A Starlike Pattern Observed in Cervical Mucus Smears Obtained From Patients With Breast Cancer and Endocrine Disorders [1]

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SUMMARY

Microscopic examination of argyrophil secretion smears from the cervical and vaginal mucus of 77 breast-cancer patients and 237 patients with illnesses other than breast cancer revealed that a starlike pattern occurs 4 times more frequently in the breast-cancer group. The percentage of occurrence of the starlike pattern was 45.2 in the breast-cancer group and 11 in the group without breast cancer. Endocrine abnormalities appeared to be associated with the occurrence of the starlike pattern.—J. Nat. Cancer Inst. 25: 1–11, 1960.

A NUMBER of characteristic physical changes in the cervical mucus is associated with the menstrual cycle. The qualitative and quantitative aspects of this phenomenon have been studied (*1–5*) and the subject has been well reviewed (*6–8*).

In addition to the physical changes, characteristic microscopic patterns have been noted (*6–11*). Most commonly observed is a fernlike pattern associated with the normal menstrual cycle. This fernlike pattern is produced by the crystallization of the cervical mucus, when dried, if adequate amounts of sodium chloride are present (*12*). It has been associated in the normal cycle with the periods of estrogen predomination [(*6–8*) figs. 1 and 2]. Atypical microscopic patterns in the cervical mucus not associated with the normal cycle or correlated with any disease have also been reported (*6, 8, 10, 11*).

In our laboratory one of these atypical patterns, a formation like a 4-pointed star, was frequently seen in the cervical mucus smears of patients with breast cancer. The following study was undertaken to evaluate the significance of this observation.

MATERIALS AND METHODS

The material evaluated was vaginal and cervical mucus smears obtained from hospital patients.

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare

The smears were air-dried, stained with a 6 percent aqueous solution of silver nitrate for 10 minutes, rinsed with distilled water, and then placed for 25 minutes in an aluminum-lined box containing two 100 watt bulbs. The smears were coverslipped with a permanent mounting medium. Staining, though not essential for microscopic detection of the mucus pattern, greatly facilitates examination.

The smears were examined under decreased light at 150×. All areas containing mucus patterns were marked (*13*) for future examination and photography.[3]

A total of 167 argyrophil secretion smears from 77 breast-cancer patients was examined. (These smears included some that had the characteristic pattern before the starlike pattern was observed.)

The hospital records of these patients were examined and the following information was recorded: patient's age, tumor type, and presence or absence of metastasis. For breast-cancer patients whose smears showed the starlike pattern, information about surgery, irradiation, hormonal therapy, and gynecologic history was also obtained.

For comparison, a group of patients was studied who had been admitted to the hospital with other illnesses during a 1-year period. Of this group, 237 had argyrophil secretion smears (a total of 308 smears) which were reviewed for this report. The clinical record of each of those patients whose smears had a starlike pattern was examined; for each, age, endocrine history, and method of treatment, including surgical procedures, were recorded. Any information relative to the breast was also noted.

RESULTS AND DISCUSSION

The crystalline pattern of dried cervical mucus resembles a 4-pointed star. The starlike formations are isolated from each other and are characterized by a right-angle intersection of the 2 axes. The density is greatest in the center and decreases toward the periphery (fig. 3). The configuration is constant, although there is an interesting but unexplained difference in size, not only from smear to smear but within each smear (fig. 4).

Papanicolaou was the first to note the starlike pattern (*11*), but did not associate it with any abnormal clinical entity. When we observed that the starlike pattern seemed to occur most frequently in association with breast cancer, the present study was undertaken to ascertain whether or not this impression was valid.

An analysis of the information obtained indicates that the starlike pattern occurs 4 times more frequently in patients with breast cancer than it does in other patients. As shown in table 1, 35 of the 77 breast-cancer patients (45.2%) had 1 or more argyrophil secretion smears showing the starlike pattern compared with 27 of the 237 (11.3%) patients

[3] The authors wish to express their appreciation to Mr. John T. Romine for the photographs used in this study.

without breast cancer.[4] When the significance of this difference was examined it was found to be significant at a probability of $P << 0.001$. To rule out the possibility that inadvertently a more intensive examination had been made of breast-cancer patients than of patients without breast cancer, a statistical analysis was made of the number of smears examined in each group. There were 167 smears from breast-cancer patients, of which 35 had a starlike pattern, and 308 smears from patients without breast cancer, of which 27 had a starlike pattern. The significance of this difference was also at the $P = 0.001$ level.[5]

TABLE 1.—Summary of cases studied by argyrophil preparation of vaginal and cervical smears

Type of case	Number of cases	Number with starlike pattern	Number without starlike pattern	Percent with starlike pattern
Patient without breast cancer	237 (308)*	27	210	11
Patient with breast cancer	77 (167)	35	42	45

*Numbers in parentheses indicate smears examined.

In seeking an explanation for this starlike pattern, it was necessary to evaluate numerous factors, such as age of the patients, type of disease, surgical procedures, and X-ray and hormone treatment, existing before the starlike pattern was observed. Tables 2 and 3 present this information for the breast-cancer patients and the patients without breast cancer, with a starlike pattern. An analysis of this data did not reveal a single definite factor or group of factors consistently associated with the pattern. However, in more general terms, the idea that hormone status undoubtedly is implicated in its formation, like hormone status in the formation of the fern pattern, is supported by the known importance of hormone therapy in breast cancer and the clinical evidence of hormone abnormality in the patients without breast cancer with a starlike pattern. In the latter group there were 6 cases of abnormal lactation, 2 cases of precocious breast development, 8 cases of hirsutism, 8 cases of amenorrhea, 3 cases of thyroid abnormality, 2 cases of adrenal dysfunction, and 4 cases of pituitary disease. Seven of the 27 patients without breast cancer with a starlike pattern were receiving thyroid hormones; 4, adrenocortical hormones; 1, posterior pituitary hormones; and 5, ovarian hormones. Only 4 of the 27 patients had no history of symptoms or treatment that might be related to the endocrine system.

The average age of the breast-cancer patients with a starlike pattern was 50 years (range 27–78 years) and the average age of the patients without breast cancer with a starlike pattern was 33 years (range 1–69 years). The starlike pattern is evidently not restricted to a particular

[4] After this study was completed, an additional 36 patients with breast cancer were examined. Fifteen of these (42%) showed the starlike pattern.

[5] The authors wish to express their appreciation to Miss Joan Gurian for the statistical analysis used in this study.

TABLE 2.—Clinical data on breast-cancer patients with starlike pattern on the argyrophil secretion smear

Hospital No.	Age	X-ray or surgery sterilization	Surgery	Hormone therapy*	Irradiation before smear
00-00-22	50	Yes	Mastectomy	2, 4	None
00-20-21	49	None	None	2, 4	Yes
00-27-98	51	Yes	Mastectomy; adrenalectomy; oophorectomy.	4, 5	Yes
00-07-51	59	None	Mastectomy	4	Yes
01-17-81	74	None	Hypophysectomy	1, 2, 5	Yes
00-19-61	49	Yes	Mastectomy; hypophysectomy	1, 5, 6	None
01-28-49	48	None	Mastectomy	3, 4	Yes
00-03-39	49	Yes	Mastectomy	2, 4	Yes
01-26-06	37	Yes	Mastectomy	None	None
00-29-47	50	Yes	Mastectomy	1, 2, 4	Yes
00-00-05	46	Yes	Mastectomy; hypophysectomy	4	Yes
01-13-59	45	Yes	Adrenalectomy	3, 4, 5	None
01-08-50	32	Yes	None	4	Yes
01-26-97	49	None	Mastectomy	None	None
01-25-27	46	Yes	Mastectomy	2	None
00-00-02	56	Yes	Mastectomy	2, 3, 4	Yes
00-06-16	52	None	None	1, 2, 3, 4, 5	None
00-16-99	47	Yes	Mastectomy	4, 5A	Yes
00-00-30	53	Yes	Mastectomy	4	Yes
00-17-72	27	Yes	Mastectomy	None	Yes
01-07-14	44	Yes	Mastectomy	1, 2	None
01-25-49	53	None	Mastectomy	2, 2A, 3, 4	Yes
01-57-11	43	Yes	Mastectomy	1, 2, 3, 5, 6	Yes
01-54-64	58	None	Mastectomy	4	Yes
01-26-83	34	Yes	Mastectomy	4	Yes
01-36-24	78	None	Mastectomy	2, 2A, 3, 5, 5A	None
01-52-50	31	Yes	Mastectomy; hypophysectomy	4	None
00-26-15	71	None	None	1, 1A, 2, 4	None
00-15-97	38	Yes	None	4, 5	Yes
00-09-84	48	None	Mastectomy	1	None
01-22-05	42	Yes	Mastectomy	4, 5	Yes
00-23-43	64	None	Mastectomy	2, 4	Yes
00-24-99	55	None	Mastectomy	2, 4	Yes
01-05-60	49	Yes	Mastectomy	3, 4	None
00-23-95	78	None	Mastectomy	1A, 2	None

*1 = Thyroid, 1A = antithyroid, 2 = estrogen, 2A = antiestrogen, 3 = progesterone, 4 = androgen, 5 = adrenocortical, 5A = antiadrenal, 6 = posterior pituitary.

age group. The relatively higher average age of the breast-cancer patients may well be ascribed to the occurrence of breast cancer in patients of this age group; consequently, breast cancer, not age *per se*, remains the distinguishing characteristic of this group.

The absence of a normal menstrual cycle was common to many of the patients, which suggested that in these patients the starlike pattern supplanted the fern pattern. Again, on the basis of individual patients, this proved unrelated to the occurrence of the starlike pattern. A number of patients with the starlike pattern did not have a history of amenorrhea or any menstrual abnormality. Nor did the presence of the starlike pattern seem to fall into a rhythmic pattern suggesting a cyclic variation similar to that associated with the normal fern pattern, which appears in the normal cycle from the 5th or 7th day through the 20th or 22d day (*6*).

The comparison between the breast-cancer patients with a starlike

TABLE 3.—Clinical data on patients without breast cancer having a starlike pattern on the argyrophil secretion smear

Hospital No.	Age	Breast change or lactation	Hirsutism	Menstrual abnormality	Hormone therapy*	Discharge diagnosis	Abortions	Surgery	Remarks
01–26–13	34	None	Marked	Amenorrhea	1, 5	Hyperadrenocorticism secondary to bilateral adrenocortical hyperplasia; Cushing's syndrome and virism	1×	Total left adrenalectomy; subtotal right adrenalectomy	None
01–25–01	37	Lactation at all times	Marked	Irregular and infrequent	None	Persistent lactation; hypertensive cardiovascular disease	None	Biopsy left breast; Dx lactation changes	None
01–82–41	44	None	Yes	Amenorrhea 4 years, then resumed menses	1, 6	Acromegaly; Graves disease	None	Thyroidectomy	Xray to skull and pituitary—30 treatments 7 years ago
00–92–92	25	After pregnancy persistent lactation	None	Amenorrhea	3, 1	Secondary amenorrhea	None	None	None
50–07–27	30	None	None	None	None	Polyarthritis	None	Benign breast tumor removed 7 years ago	None
00–78–28	30	Slight, persistent lactation	None	Irregular	None	Mild anxiety reaction	4×	None	None
00–86–13	36	None	Yes	Oligomenorrhea	None	Hirsutism	None	None	None
01–64–28	34	None	None	Amenorrhea	1	Chromophobe adenoma pituitary	None	Hypophysectomy	None

See footnote at end of table.

TABLE 3.—Clinical data on patients without breast cancer having a starlike pattern on the argyrophil secretion smear—Continued

Hospital No.	Age	Breast change or lactation	Hirsutism	Menstrual abnormality	Hormone therapy*	Discharge diagnosis	Abortions	Surgery	Remarks
01–62–72	38	Slight lactation	None	Amenorrhea	5, 1 Growth hormone	Anterior pituitary insufficiency	None	None	None
00–86–02	51	None	None	Amenorrhea	None	Choriocarcinoma; dermatitis; herpetiformis	1×	Hysterectomy; oophorectomy	Methotrexate treatment
00–91–41	35	None	None	None	Steroid type	Chorioretinitis; amblyopia; duodenal ulcer	None	Cyst from ovary	None
00–88–83	34	None	None	Dysmenorrhea	None	Mental deficiency; congenital lues	None	None	None
01–98–30	69	None	None	None	1	Thyroiditis	None	None	None
00–93–70	45	None	Yes	None	5	Adrenocortical carcinoma	None	None	None
01–88–09	28	None	None	None	None	Tetralogy of Fallot	3×	Right oophorectomy	None
01–96–49	45	None	None	None	None	Carcinoma of cervix	None	None	Radiation
01–78–43	32	None	None	Amenorrhea	3, 2, 5	Secondary amenorrhea	2×	None	Skin pigmentation
01–85–60	2	Breast development	None	None	None	Precocious breast development	None	None	None

01–42–29	43	None	None	Metorrhagia	None	Malignant melanoma	4×	Hysterectomy; salpingectomy; left oophorectomy; axillary dissection	None
00–81–34	20	Persistent lactation	None	Intermenstrual spotting	2	Chorioadenoma destruens	None	Pan hysterectomy	None
00–80–47	28	Persistent lactation	Yes	Amenorrhea	1, 2	Lactation; hirsutism	None	None	None
01–41–90	37	None	None	None	1A	Hyperthyroid	None	Tubal ligation	None
00–80–49	1	Breast enlargement	Excess pubic hair	None	None	Precocious puberty	None	None	None
01–96–51	35	None	None	Dysmenorrhea	None	Heart disease	1×	Uterine suspension	None
02–03–73	21	None	Yes	Irregular	None	Hirsutism	None	None	None
00–92–91	60	Breast lumps	None	None	None	Diabetes mellitus	None	Hysterectomy	None
00–89–47	40	None	None	Early menopause	2	Functional neurocirculatory asthenia	None	None	None

*1 = Thyroid, 1A = antithyroid, 2 = estrogen, 2A = antiestrogen, 3 = progesterone, 4 = androgen, 5 = adrenocortical, 5A = antiadrenal, 6 = posterior pituitary.

pattern and the breast-cancer patients without a starlike pattern was also interesting. The average age of breast-cancer patients with a starlike pattern was 50 years. The age range for this group was 27 to 78 years. For the breast-cancer patients without a starlike pattern the average age was 51 years with a range from 28 to 75 years. Thirty-one of the 35 patients with a starlike pattern had metastases and 30 of the 42 patients without a starlike pattern had metastases. There was also an approximately equal distribution of cases with and without the starlike pattern among the various histologic types of breast cancer. No obvious common factor or group of factors related to surgery, irradiation, hormone therapy, or gynecologic disorders were associated with occurrence of the starlike pattern in these breast-cancer patients (table 2).

Most breast-cancer patients were in the postmenopausal age group. It has been previously observed that women of this age do not normally show crystallization of the cervical mucus (*6–8*). Consequently the occurrence of any consistent pattern in approximately half the patients in this age category is, in itself, interesting. As might be anticipated on the basis of previous studies, most smears from breast-cancer patients without a starlike pattern have no pattern.

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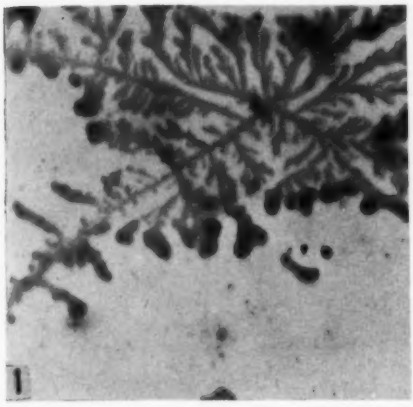
PLATE 1

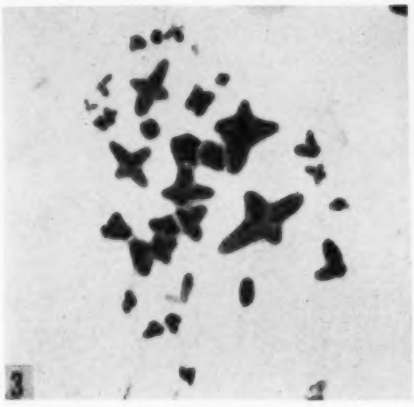
FIGURE 1.—Pattern seen in cervical mucus during follicular phase of normal menstrual cycle. Stained with silver nitrate. × 245

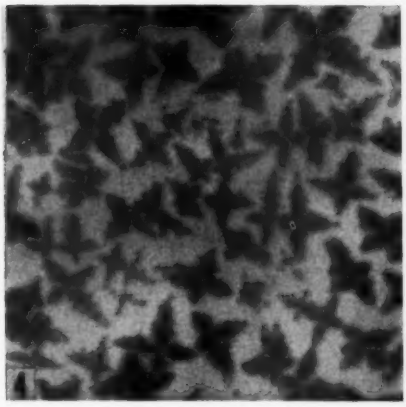
FIGURE 2.—Pattern seen in cervical mucus at time of ovulation of normal menstrual cycle. Stained with silver nitrate. × 245

FIGURE 3.—Starlike pattern seen in cervical mucus from patient with breast cancer. *Note* starlike shape with decreasing density of material toward periphery. Stained with silver nitrate. × 280

FIGURE 4.—Starlike pattern seen in cervical mucus from patient with breast cancer. *Note* variations in size of starlike formations. Stained with silver nitrate. × 535







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An Evaluation of the Proposed International Clinical Staging System for Cancer of the Breast [1,2]

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SUMMARY

The Committee on Clinical Stage Classification and Applied Statistics of the International Union Against Cancer has proposed a clinical staging system for breast cancer. To evaluate the error involved in the use of this 4-stage system, we have applied it to each of 671 cases as a clinical and also as a "final" system, *i.e.*, one with all available information, including that from surgery and pathology. Inspection of survival curves showed that the proposed system defined 4 distinct stages when applied as a final system. When applied as a clinical system the curves for stages I and II were not clearly separate from one another, nor were the curves for stages III and IV. Frequent sources of error in clinical examination which were important to staging related to (*a*) state of homolateral axillary nodes in stages I and II, (*b*) size of primary tumor in stage III, and (*c*) state of nodes other than homolateral axillary nodes in stage IV. Error (*c*) was associated with the least experienced examiners. When the clinical staging was corrected for error (*c*), which presumably would not occur when the most experienced examiners are managing a series of cases, the distinction in the survival curves for the various stages was considerably improved. After this correction it was still noted, however, that 39 percent of clinical stage I cases were final stage II, 34 percent of clinical stage II cases were final stage I, and 52 percent of clinical stage III cases were either final stage I or II. The use of the clinical system in therapeutic trials was considered. It was concluded that in spite of its errors the clinical staging system may be used under various conditions to estimate the magnitude of differences in therapeutic effect that would be observed in the absence of errors in staging.—J. Nat. Cancer Inst. 25: 13–24, 1960.

FOR A valid comparison, by stage, of the survival of cancer patients treated in different ways, it is necessary (although not necessarily sufficient) to use a staging system based on information equally available for all treatment groups. Consider the situation where radiation or chemotherapy is to be compared with surgery. Obviously, the information potentially available for staging the nonsurgical cases is considerably

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[2] This study was supported in part by the End Results Evaluation Section, Cancer Chemotherapy National Service Center, National Cancer Institute, and by the U.S. Atomic Energy Commission, Contract AT-11-1-GEN-10, No. 2.

[3] The authors are greatly indebted to Mrs. Marilyn Vaage for her technical assistance.

less than that for the surgical cases, and if all available information were used, the surgical cases would be the more accurately staged. To put treatment groups such as these on a common basis, *clinical* staging systems have been devised. Such a clinical system for cancer of the breast has been recommended by the Committee on Clinical Stage Classification and Applied Statistics of the International Union Against Cancer (*1*). With the physician's findings (including roentgenologic evidence) on the extent of the tumor at the primary site, the regional lymph nodes, and other distant organs, a case is placed in one of four stages, to be defined later in this paper.

The cases of carcinoma of the breast seen at the University of California Medical Center, San Francisco (1942–56), were staged by us according to the Committee's system, both on a clinical and on a "final" basis. "Final" as used here implies the use of all information available at the end of the first course of treatment or at the time of the patient's first discharge from the hospital, including the surgical and histopathologic data as well as that used in the clinical appraisal. In addition, these cases are routinely classified on the basis of a conventional 3-stage "final" system by the General Tumor Registry of the Cancer Research Institute at this Medical Center.

This paper is primarily concerned with 4 questions: When the 4-stage system proposed by the Committee is used, to what extent do "clinical" evaluations differ from "final" ones? What are the principal causes of the differences? What advantage has the proposed system over a conventional 3-stage system, especially when the resulting survival curves are compared? How do errors in clinical staging affect the comparative evaluation of 2 forms of therapy?

METHODS AND CASE MATERIAL

Staging procedures.—To classify each case, the hospital unit record was reviewed and the pertinent facts were recorded on a specially designed abstract sheet. In addition to the clinical, surgical, histologic, and radiologic findings, the nature of the treatment, and a record of follow-up were entered. When the record was inconsistent, with respect to a particular clinical finding, the opinion of the senior examining physician was accepted.

The staging system proposed by the Committee [4] is intended for the findings in previously untreated patients examined clinically, *i.e.*, by general physical examination, diagnostic roentgenography, and other methods in which instruments are available to the average specialist for clinical diagnosis. Surgical and histologic examination are excluded. The cases thus classified will be referred to as in a particular *clinical stage*.

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[4] The Committee's Report may be obtained by writing to Dr. P. F. Denoix, International Union Against Cancer, 25, Rue d'Ulm, Paris-5e, France.

The criteria for clinical staging have been given in detail (*1*). The positive criteria, summarized briefly, are the following:

Stage I: Tumor 5 cm. in diameter or less. Skin fixation absent or incomplete. Nipple may be retracted or Paget's disease may be present.

Stage II: Findings same as in stage I. In addition, palpable, movable, homolateral axillary nodes are present.

Stage III: Tumor more than 5 cm. in diameter, or skin fixation complete, or skin involvement wide of tumor, or orange-peel skin present, or pectoral muscle fixation, or chest-wall fixation present, or homolateral axillary nodes fixed, or homolateral infra or supraclavicular nodes present.

Stage IV: Distant metastases present.

Every case was also classified by this system on the basis of *all* available information, including reports by the surgeons and pathologists. The clinical findings were not accepted unless confirmed by the surgical and microscopic findings. Thus, for example, the definitive size of the tumor used in the staging was that estimated by the surgeon or measured by the pathologist; the presence of positive nodes was taken from the diagnosis of the pathologist. The evidence of radiography for distant metastases was used in the final staging as well as in the clinical. The cases thus classified will be referred to as in a particular *final stage*.

The routine system of final classification of the General Tumor Registry, designed to be consistent with the recommendations of the California Tumor Registry, California State Department of Health, used the following criteria:

Stage I: Tumor confined to breast, as indicated by histologic examination of the breast, the surrounding tissues, and the homolateral nodes.

Stage II: Tumor as in stage I, and involvement of the chest wall, or of homolateral lymph nodes, or of cutaneous lymphatics, proved by histologic examination. Or the presence of skin nodules whose nature need not be histologically proved.

Stage III: Involvement of supraclavicular, heterolateral axillary, or cervical lymph nodes established histologically. Or other distant metastases present as proved by such methods as radiography, biopsy, and bronchoscopy.

The cases thus classified will be referred to as in a particular *final stage* (GTR).

Case material.—The 1,334 patients with malignant breast tumors seen at the University of California Medical Center, 1942–56, inclusive, and listed in the General Tumor Registry, were reviewed. Of these cases, 671 women (96% white) were found suitable for the present study, *i.e.*, they were examined and treated (for the first time) at the Medical Center, in such a way that a precise comparison could be made between clinical and final staging. Thus the pretreatment record contained the necessary statements regarding such findings as primary tumor, axillas, and distant metastases, and the surgery and pathology reports likewise dealt specifically with the same items. Table 1 gives the age distribution of the total group (1,334 cases), which served as the standard population for various age-adjustment procedures, and that of the study group (671 cases).

TABLE 1.—Distribution of breast carcinoma by age of patient

Population	All ages	35 (under 40)	45 (40–49)	55 (50–59)	65 (60–69)	75 (over 69)
	Age group* (*years*)					
Standard population† (1, 334 cases)	1. 00	0. 14	0. 27	0. 26	0. 21	0. 12
Study group (671 cases)	1. 00	0. 11	0. 25	0. 28	0. 23	0. 13

*Age at time case was diagnosed.
†All cases during 1942–56, inclusive.

The distribution of treatment in the study group is given in table 2. As would be expected, the treatment did not vary greatly in stages I, II, and III of the 4-stage system. In general, all patients who could tolerate the treatment were subjected to radical surgery and in addition, depending on the policy of the individual surgeon in consultation with the radiation therapist, about half the patients in stages II and III also received X-ray treatment. In stage IV, for obvious reasons, only 14 percent of the patients received radical surgery.

TABLE 2.—Distribution of treatment (%) by final staging

Treatment	Stage I	Stage II	Stage III	Stage IV
Surgery only				
Radical	79	52	39	7
Simple	3	3	9	9
Surgery + X ray				
Radical	16	41	40	7
Simple	2	4	10	19
X ray only			2	20
Other				38
Total:				
Percent	100	100	100	100
Number of cases	229	245	140	57

The follow-up period for the series was closed June, 1958. For cases in the General Tumor Registry, successful follow-up exceeds 95 percent.

Some of the material in the present study was reported previously by Shimkin *et al.* (*2*) in their analysis of cases in 1918–47, inclusive. The two studies complement one another in a general way; the objectives of the earlier study were different from those of the present one, and the final staging system differed from the 4-stage system used in the present study. The study by Shimkin *et al.* dealt specifically with the influence on survival of the interrelated factors of age at onset, age at treatment, and stage of disease; the data were compared with results from four other centers.

Calculation of age-adjusted probability of survival.—Age-adjustment techniques should be used in comparing the survival of groups that differ in age. The adjustments in the present study assumed that each group had the age distribution of the 1942–56 total pool of 1,334 cases (standard population) at diagnosis. For purposes of computation, this standard population was divided into 5 age categories whose relative numbers at zero time are given in table 1.

To determine the age-adjusted cumulative survival curve, the following steps were carried out:

1. The particular group under study, *e.g.*, stage I, was divided into the 5 age categories defined for the standard population.

2. For the cases in each age category, the actuarial method of Berkson and Gage (*3*) was used to obtain values of q_x, the probability of dying in the time interval x to $x + 1$ years after diagnosis (given that the individual had survived to the beginning of that interval).[5] Values of p_x, the probability of surviving the specified interval, were obtained by subtracting q_x from 1.

3. The age-adjusted probability of dying within the period x to $x + 1$ years after diagnosis, $\bar{q}_x$, is obtained by computing

$$\bar{q}_x = 0.14q_{x_{35}} + 0.27q_{x_{45}} + 0.26q_{x_{55}} + 0.21q_{x_{65}} + 0.12q_{x_{75}}. \quad (1)$$

Here the weighting factors (0.14, 0.27, etc.) are the proportions of the standard population falling into each of the 5 age categories (table 1). The q_x values in the formula of equation 1 are the specific values for each age group whose midpoint (real or assumed) is indicated in the subscript, *e.g.*, $q_{x_{45}}$ is the value for the age group 40 through 49 years. The corresponding age-adjusted probability of survival, $\bar{p}_x$, is equal to $1 - \bar{q}_x$.

4. The age-adjusted probability of surviving to $x + 1$ years after diagnosis, also referred to as the cumulative probability of survival, is given by

$$\bar{P}_{x+1} = \bar{p}_0 \cdot \bar{p}_1 \cdot \bar{p}_2 \cdot \ldots \bar{p}_x. \quad (2)$$

To obtain the expected survival curve for a comparable "control" group in the general population, the procedure described was used. The values of q_x were obtained from the tables of age-specific mortality data for the white female population of the United States, 1949–51 (*4*).

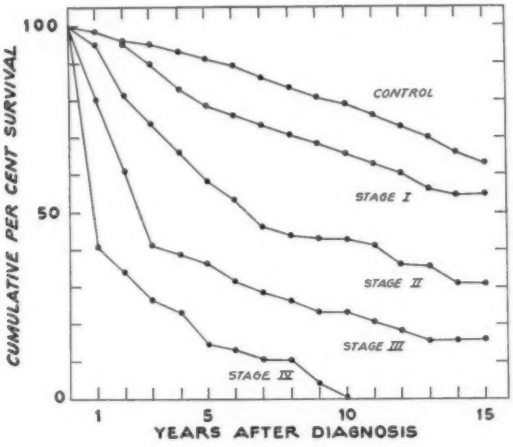
RESULTS AND DISCUSSION

Comparison of Survival Curves

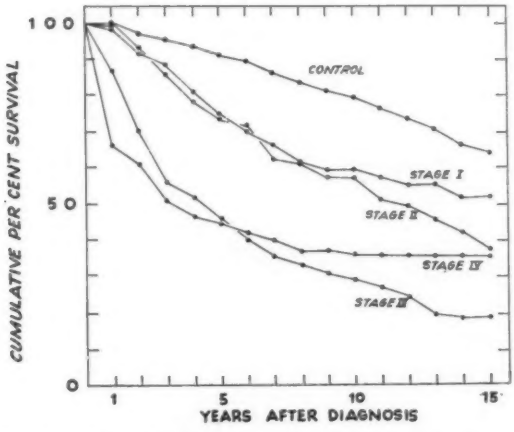
Examination of the survival curves showed that the proposed international system defined 4 distinct stages when it was applied as a final

[5] q_x=number dying during the period x to $x+1$ years after diagnosis/number alive at year x minus one half the number of cases lost to study during the period year x to $x+1$. Cases lost to study are: (*a*) cases lost to follow-up and (*b*) cases (patient still alive) being followed but were first seen less than $x+1$ years ago and therefore could not contribute survival information for year x to $x + 1$.

system (text-fig. 1) but not when applied as a clinical system (text-fig. 2). Clinically stages I and II were distinguishable from stages III and IV. However the curves for stages I and II overlapped considerably during the first 10 years after diagnosis. The curves for stages III and IV crossed one another at 5 years after diagnosis, with the survival for stage IV remaining unusually high from that point on.



TEXT-FIGURE 1.—Age-adjusted survival curves for cases of cancer of the breast (and for a control group) staged by the *final* 4-stage system. In this and the following graphs, the control group is based on data for the U.S. population of white females, 1949–51. The age adjustment is based on the distribution of ages in the "standard population" (table 1).



TEXT-FIGURE 2.—Age-adjusted survival curves for cases of cancer of the breast staged by the *clinical* 4-stage system.

Errors in Clinical Staging

Specific causes of the disagreement between clinical and final staging were sought by comparing the results of clinical, surgical, and histologic examinations (table 3). The condition of the axillary nodes was frequently misjudged clinically. In clinical stage I, 40 percent of the cases had positive nodes; in stage II, 38 percent had negative nodes. In stage III, the tumor size was overestimated in 33 percent of the cases, and its extension into neighboring organs was misjudged either positively or negatively in about 12 percent. Also, in 18 percent the axillary nodes were incorrectly judged to be positive. It should be noted that for cases in which 2 or more of the criteria for clinical stage III were judged positive, an error in one of them would not change the final staging.

TABLE 3.—Frequent sources of error in clinical examinations

		Source of error†		
		Primary site		Homolateral axillary nodes
Clinical stage*	Cases (*total*)	Tumor size	Extension to neighboring organs	
		Number of errors		
I	201	2	3	80
II	111	1	0	42
III	249	83	29	44

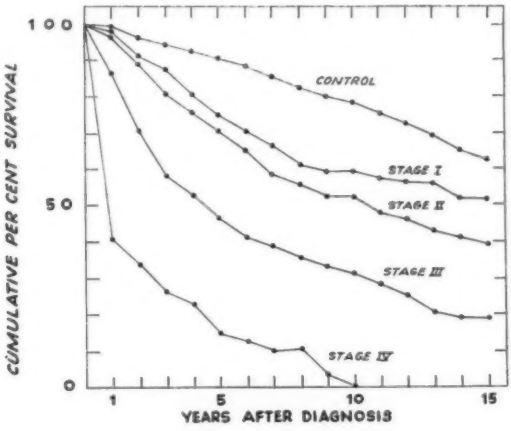
*For clinical stage IV, *see* table 4.
†In some cases there was more than 1 error.

These results were not altogether unexpected, since the difficulties in assessing the axillary nodes and also in estimating tumor size are common experiences. Thus at Radiumhemmet in Stockholm where 3 senior men examine the patient (1 surgeon and 2 radiologists), 28 percent of the cases clinically judged negative for axillary node metastasis were histologically positive, and 15 percent of the clinically positive cases were, in fact, negative (*5*). In a survey of 2,400 cases in the literature, Nohrman (*6*) found that 44 percent of cases clinically judged negative were histologically positive, and 14 percent of the clinically positive cases were negative. In the present series, therefore, the error in clinical stage I (40%) might have been predicted from the literature. The combined error in clinical stages II and III (86 cases in 360 = 24%) was greater than usual.

A particular group of cases was responsible for the peculiarly good result in clinical stage IV. Of the 671 cases in the series, 59 depended critically for staging on the presence of carcinoma in the heterolateral axillary nodes (49 cases), the supraclavicular nodes (6 cases), or other distant lymph nodes (4 cases). In each of the 59 cases, a junior physician had judged the nodes clinically to be positive, but from the case record it could be inferred that the diagnosis was not accepted by the operating surgeons and by others who reviewed the case, since no steps were taken to perform a biopsy or to excise these nodes, nor were they considered at any time in the subsequent discussion of the case. These 59 cases (53, stage IV; 6, stage III)

were therefore restaged clinically by us on the assumption that the nodes in question were negative.

The effect of the restaging on the distribution of cases is shown in table 4. The table shows the distribution for the uncorrected group of 612 cases (line 1) and the distribution of the 59 cases before correction (line 2a) and after correction (line 2b). The effect of restaging was to shift the majority of these cases from clinical stage IV to stage II. (Their final staging, of course, was unaffected.) The survival curves (text-fig. 3) then indicated clearly that the prognosis of the corrected clinical stages followed the numerical order of staging.



TEXT-FIGURE 3.—Age-adjusted survival curves for cases of cancer of the breast by corrected clinical stage. The correction involved restaging 59 cases after a review of the hospital records indicated clinical diagnosis of cancer in distant lymph nodes to be incorrect. We suppose such curves to represent the type of result obtainable when cases are staged by senior men.

TABLE 4.—Distribution of cases by clinical (C) and final (F) stage (proposed international system) before and after correction for 59 cases with the erroneous diagnosis of distant lymph-node metastasis

Group*	Stage (*number of cases*)								Total No. of cases
	I		II		III		IV		
	C	F	C	F	C	F	C	F	
1. Uncorrected	201	203	111	220	243	132	57	57	612
2. Clinical diagnosis corrected:									
a. Before correction	0	26	0	25	6	8	53	0	59
b. After correction	4	26	39	25	16	8	0	0	59
Total (1+2a) uncorrected†	201	229	111	245	249	140	110	57	671
Total (1+2b) corrected‡	205	229	150	245	259	140	57	57	671

*For further definition of groups, *see* text.
†Clinical stages used for text-figure 2.
‡Clinical stages used for text-figure 3.

The relation between corrected clinical stage and final stage in individual cases was revealed by the 2-way classification of cases in table 5. For clinical stages I to III, only about 55 percent of the cases so diagnosed received the same final diagnosis. The table showed that 39 percent of cases in clinical stage I were, in fact, final stage II, that 34 percent of cases in clinical stage II were final stage I, and that 52 percent of cases in clinical stage III were either final stage I or II. Only clinical stage IV was diagnosed accurately (98%). The failures in clinical diagnosis in stages I and II resulted from errors in assessing the state of the axillary nodes (table 3), a difficulty in all systems of classification. In addition, clinical judgment frequently oversized the tumor at the primary site, a matter of particular importance for stage III in the international system. Judged by the comparison of curves in text-figure 3 with those in text-figure 1, these errors lowered the survival of stage I and raised that of stage II, thus largely obliterating the difference in the prognosis of the two stages. The errors also tended to raise the survival of clinical stage III.

TABLE 5.—Distribution of cases by corrected clinical stage and by final stage

Corrected clinical stage	Final stage				Clinical cases:	
	I	II	III	IV	Total	Percent
I	117 (57%)	80 (39%)	8 (4%)		205	31
II	51 (34%)	92 (61%)	7 (5%)		150	22
III	61 (24%)	72 (28%)	125 (48%)	1 (0%)	259	39
IV		1 (2%)		56 (98%)	57	8
Final cases:						
Total	229	245	140	57	671	100
Percent	34	37	21	8		100

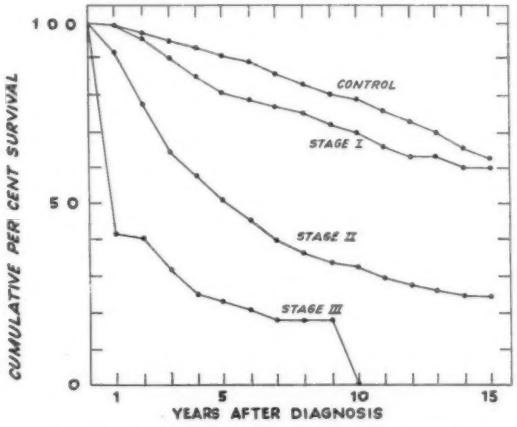
Presumably, the discrepancy between clinical and final staging can never be eliminated, owing to the impossibility of accurately judging the axillary nodes clinically. The considerable percentage of errors still remaining for corrected clinical stages I and II cases accounts for the proximity of the survival curves for these two stages in text-figure 3 as compared with comparable curves based on final staging in text-figure 1.

Since roentgenologic evidence was used as acceptable criteria for both clinical and final staging, it was to be expected that most corrected clinical stage IV cases would also be in final stage IV. The corrected data represented, or presumably at least approximated, what senior examiners would find. Harmer (7) has pointed out in a constructive discussion of the proposed international system that the accurate clinical diagnosis of stage IV is of particular importance, since it is now generally agreed that such patients should not receive radical surgery. This, of

course, is in contrast to stages I to III for which surgical treatment with intent to cure is indicated.

Three-Stage Versus Four-Stage Systems

When the GTR 3-stage (final) system was used, the survival curves (text-fig. 4) showed 3 stages that were clearly different from one another. However this 3-stage system was considered inferior to the final stage classification based on the international system, since the latter clearly defined 4 stages. Stage I was practically the same in both systems. Stage II (GTR) for the most part averaged the data of stages II and III of the international system. Stage III (GTR) comprised chiefly the cases in stage IV and a few in stage III of the international system. Shimkin *et al.* (*2*) also found that the 4-stage final system of Portmann (*8*) clearly defined 4 stages in their study of the 1918–47 series of cases seen at this Medical Center.



TEXT-FIGURE 4.—Age-adjusted survival curves for cases of cancer of the breast staged by the final 3-stage system.

Clinical Staging and Therapeutic Trials

The bearing of the present findings on the design of therapeutic trials requires some discussion. When extensive surgical examination is not carried out, as in radical radiation or chemotherapy, and comparison of the merits of 2 forms of therapy is desired, the cases must be staged on a clinical basis before they are assigned to a treatment group by a system of randomization. To what extent do errors in clinical staging diminish the sensitivity of such a trial based, for example, on survival at 5 years? To answer this question, let us consider a trial in which a new form of treatment N is compared with a standard form S. The following information is needed:

(*a*) The 5-year survival for correctly staged cases given treatment *S*. We may use for illustrative purposes the data represented in text-figure 1: stage I, 78 percent; stage II, 58 percent; stage III, 37 percent. Stage IV will not be considered.

(*b*) The 5-year survival for correctly staged cases given treatment *N*. This will depend on how effective *N* is assumed to be. Four sets of assumptions have been made (conditions 1–4 in table 6), and the results of each will be discussed in turn.

TABLE 6.—Treatment comparisons: results with clinical and final staging

Conditions	Treatment	5-year survival (%) Stage I	Stage II	Stage III
1. No difference between N and S	N	78* (69)†	58 (64)	37 (53)
	S	78* (69)†	58 (64)	37 (53)
	N – S	0‡ (0)§	0 (0)	0 (0)
2. N better by 10% at each stage	N	88 (79)	68 (74)	47 (63)
	S	78 (69)	58 (64)	37 (53)
	N – S	10 (10)	10 (10)	10 (10)
3. N better by 20% at stages I and II	N	98 (88)	78 (83)	37 (63)
	S	78 (69)	58 (64)	37 (53)
	N – S	20 (19)	20 (19)	0 (10)
4. N better by 20% at stage I and by 10% at stage II	N	98 (84)	68 (77)	37 (60)
	S	78 (69)	58 (64)	37 (53)
	N – S	20 (15)	10 (13)	0 (7)

*Final stage.
†Clinical stage.
‡True difference in survival.
§Difference in survival based on clinical staging.

(*c*) The 5-year survival for clinically staged cases given treatment *S*. The distribution of final (*i.e.*, correct) stages within each clinical stage is given in table 5. From these data and those given under (*a*) the 5-year survival of clinically staged cases is calculated.[6]

(*d*) The 5-year survival of clinically staged cases given treatment *N*. This will depend on which set of assumptions is applied.

If the clinical staging system reflects perfectly the difference in effectiveness of the 2 methods of treatment, the difference in the values for (*a*) and (*b*) will agree exactly with the difference between (*c*) and (*d*) for each stage. Table 6 shows such comparisons under 4 sets of assumptions regarding the efficacy of the new form of treatment.

[6] Each clinical-stage group is a composite of individuals from the various final stages. For a particular clinical stage, survival is the weighted average (weights from table 5) of survival for the 3 final stages, *e.g.*, for clinical stage I treated by method *S* the expected survival is (0.57)(78) + (0.39)(58) + (0.04)(37) = 69 percent. This value of 69 percent does not agree with the result (74%) seen in text-figure 3. For agreement between these 2 results, it would be necessary for each age-specific risk of dying in equation (1) to represent a weighted average of the risks for the various final stages entering into a clinical stage group. For stage I the weights would again be equal to 0.57, 0.39, and 0.04, as given in text.

Condition 1.—When the new treatment is neither more nor less effective than the standard one, both clinical and final staging demonstrate this fact equally well.

Condition 2.—When the new treatment improves survival at each stage by the same amount, *e.g.*, 10 percent (absolute), both clinical and final staging demonstrate this fact equally well.

Condition 3.—When the new treatment affects the stages differentially, clinical and final staging do not give the same results. In the present instance, the new treatment improves the results by 20 percent in stages I and II, but has no effect in stage III. Clinical staging reflects closely the effect in stages I and II but gives a false positive in stage III owing to clinical stage III's contamination with true cases of stages I and II.

Condition 4.—This illustrates an instance in which the differential action of the treatment leads to an underestimation of efficacy in one stage and an overestimation in another. This situation assumes that the new treatment is better than the standard by 20, 10, and 0 percent at stages I, II, and III, respectively.

These situations, although hypothetical, are not improbable and might occur in the testing of a drug for breast cancer. It is apparent from this study that trials based on clinical staging may provide satisfactory or, at least, useful estimates of the results that would be obtained without errors in staging.

Under the conditions cited, it is assumed either that the new therapy has no effect on survival at a given stage or that the effect is positive. Complications in evaluation arise when the effects are positive in some stages and negative in others. The largest errors under conditions 3 and 4 are observed in estimating the effect at stage III in which there is assumed to be no real effect. Of the two errors—overestimating or underestimating the true effect at stage III—it is the former and probably less costly error which is made.

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The Micromorphology of a Salivary-Gland Tumor of a Mouse Infected with Polyoma Virus [1,2]

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SUMMARY

A salivary-gland tumor from a mouse infected at birth with polyoma virus was studied with the electron microscope. Intranuclear, intracytoplasmic, and intercellular particles were observed. Intranuclear particles average 30 mμ and appear to be associated with fine filamentous material. Viruses of 80 mμ similar in structure to leukemia viruses were seen at the cell borders. Both plasma membrane and filamentous material in the cortical cytoplasm appear involved in their formation. Cytoplasmic virus particles associated with mitochondria and inclusion bodies are of size and structure similar to the intranuclear particles. Crystalline aggregates may occur in the inclusion body. Intercellular virus particles include dense forms of 30 mμ identified as polyoma virus, double-membraned forms of 80 or more mμ containing a nucleoid and tentatively identified as leukemia virus, single-membraned forms of 60 to 70 mμ containing an eccentric nucleoid such as the mammary-tumor agent contains, although smaller than the latter, and single-membraned forms of 65 to 70 mμ containing unidentified particles and vesicles. Smaller vesicles of 33 to 52 mμ also occur in the peripheral cytoplasm and intercellular spaces. Their relation to the tumor viruses is undetermined.—J. Nat. Cancer Inst. 25: 25–51, 1960.

CELL-FREE extracts derived by Gross (*1*) from leukemic AK mice, when injected into newborn mice, cause not only leukemia but also a variety of tumors, those of the salivary gland being the most conspicuous (*2*, *3*). There are probably 2 viruses involved (*4*)—the L (leukemia) virus and the P (polyoma) virus. Recently several methods have been used to characterize these viruses and to determine the relationship between the two, if any. With the electron microscope, the P virus has been studied in tissue cultures (*5–8*), in tumors (*5*, *9*), and in the spleen of infected mice (*5*). Particles presumed to be similar to the L virus have been observed in tumors of infected mice (*10*). A second type of particle different from the P virus, but similar to the type seen in other mouse and human leu-

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[2] This work was aided by grant P-145B from the American Cancer Society, Inc., and grant C-4926 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] Deceased, March 1, 1960.

kemias (*11*), has also been seen in cultures inoculated with the parotid-tumor agent (*6*).

In the present study a salivary-gland tumor from a mouse infected at birth with P virus was studied in thin sections with the electron microscope. The results in part confirm and in part extend previously published results on the polyoma virus. Intranuclear, intracytoplasmic, and intercellular particles were observed. There appear to be several types of particles in a given tumor cell, and perhaps as many as 4 different viruses within the tumor.

MATERIALS AND METHODS

The polyoma virus preparation was obtained from 4 spontaneous AK mouse leukemias that were carried by transplantation through 4 to 5 serial subpassages. Leukemic tissues were collected from the final subpassages, and fractions were prepared by the nitrogen-bomb technique (to be published) and subsequent differential centrifugation, as described previously (*4*). The fractions were stored at −70° C. until recipients were available.

Figures 1 through 24 are micrographs of a salivary-gland tumor that developed in an AK × $C3HF_1$[4] hybrid female mouse. This animal had bilateral salivary-gland tumors, multiple mammary tumors, microscopic kidney tumors, and P lesions in the thymus, thyroid, and submucosal glands of the trachea. There was no evidence of leukemia.

The animal was killed 144 days after infection. It had been injected at less than 12 hours of age with the supernatant fluid obtained by recentrifugation, at 105,000 × g for 2 hours, of the supernatant fluid that had been centrifuged at 105,000 × g for 35 minutes. The tissue was prepared by standard procedures with osmium tetroxide fixation and methacrylate embedding. Sections were cut with a Fernández-Morán diamond knife on a Porter-Blum microtome and observed in the Siemens' Elmiskop I.

RESULTS

General

The salivary-gland tumor is characterized by cells possessing large nuclei and small cytoplasms (fig. 1). In the electron microscope the cells are seen to make intimate contact at only few places along their borders. The cell borders vary from smooth to rugose or microvillate. Basement membrane material is absent, except in the interspace of the infrequent desmosomes. The desmosomes are short and show either microvesicles and fine filaments extending into the cytoplasm (fig. 15) or a doubling of the cell membrane. Filamentous material may occur in the interstitium.

[4] C3Hf/BiGsLv—indicating C3H mice, foster nursed, originally obtained from Bittner, carried for several generations by Gross, and then Gross' subline carried by Levinthal.

The nucleus (fig. 1) of the tumor cell generally is irregularly ovoid. It is bounded by 2 membranes (fig. 2), the outer one often continuous with membranes of the endoplasmic reticulum. Nuclear pores are frequent, often showing granular material apparently continuous from nucleoplasm to cytoplasm. The chromatin is peripheral and the several large nucleoli are generally eccentric. The nuclear matrix is densely granular and filamentous.

The contents of the small cytoplasm vary considerably from cell to cell. The matrix is characterized by numerous RNP (ribonucleoprotein) particles (fig. 11) both free and attached to membranes of the endoplasmic reticulum. The endoplasmic reticulum exists in both the smooth (fig. 7) and rough profiled forms (figs. 9 and 11). The Golgi apparati (fig. 1) are large, complex, and scattered. They comprise dictyosomal, microvesicular, and macrovesicular elements and multivesicular bodies. Fine to coarse filaments occur in the peripheral cytoplasm of many of the cells. The mitochondria are variable in size and shape. Characteristically they are large, irregular, and have few but distinct cristae and a dense matrix of fine granules.

Intranuclear Virus Particles

Intranuclear virus particles were observed in a large number of the tumor cells. Generally they were located in scattered, clear areas of the nucleus (figs. 1 and 2). In a few cells almost the entire nucleus was occupied by viral particles (fig. 3). Commonly the particles were intimately associated with filaments in the nucleoplasm, and the filaments and associated particles often appeared to be in contact or continuous with the denser filamentous material at the periphery of the nucleolus (fig. 5). Viral particles were not observed within the nucleolus itself, however.

The individual nuclear viral particles were essentially uniform in diameter ranging from 26 to 33 mμ, with an average of 29.6 mμ. There appeared to be considerable variation in structure, however, not easily explained by the plane or thickness of the section. At low magnification (fig. 3) some particles appeared dense and homogeneous; others looked hollow or as if they contained one or more dense inner particles. At high magnification (fig. 4) all particles were similar. The variations observed were in the density of filamentous material comprising the particle. Component threads seen in regular alignment in some particles or at the fringes of less regularly composed particles averaged 14 Å in thickness. Their length could not be determined. The best evidence from all micrographs examined to date indicates that the particle is in effect a loosely wound mass of threads.

Cytoplasmic Virus Particles

Two sites of intracytoplasmic virus particles have been observed in the tumor cells. The first of these is in mitochondria and inclusion bodies (figs. 6 through 12) in the inner cytoplasm. A second site, containing large particles and small membrane-limited vesicles, occurs at the modified

border of the tumor cell (figs. 13 through 24). The several types of viruses may be seen within one cell or may occur separately in different cells from the same area of the tumor.

To speculate upon the origin and the nature of the particles associated with the inclusion bodies, it is first necessary to describe the inclusion body itself and to relate the probable steps in its formation. The best evidence indicates that the inclusion bodies originate from the mitochondria. Structures are observed which range from normal-looking to modified mitochondria. In addition, there are inclusion bodies which range from small double-membrane-limited structures of a size similar to mitochondria up to structures often one fourth the size of a nucleus, but with vestigial membranes suggestive of mitochondrial outer membranes and cristae.

The normal mitochondria of these cells are irregular in shape, roughly cylindrical, ranging up to 780 mμ in length and averaging 250 mμ in width in the plane of sectioning. They posses few, incomplete cristae. The membranes of the cristae and the limiting membranes of the mitochondria are quite distinct. In those cytoplasmic areas in which inclusion bodies are seen, the mitochondria are small, averaging 150 mμ × 240 mμ, roughly spherical to ovoid, and have only a few cristae. The outer membranes of such mitochondria are denser than normal, and the matrix is full and finely filamentous (figs. 7, 8, and 11). Other bodies in the same area are slightly larger than the mitochondria described and have a dense matrix, no cristae, but distinct outer membranes. These bodies appear to be modified mitochondria. Still larger structures roughly spherical and with double-limiting membranes may be seen in the same areas. However, the limiting membranes of such structures are not as distinct as those of the smaller bodies and modified mitochondria. In some of these membrane-limited structures (figs. 9 and 10), vestiges of inner membranes may be seen in the dense matrix suggesting that these bodies, herein designated as inclusion bodies, are derived from either enlarged or aggregated mitochondria. Some forms have been observed (not illustrated) in which the large inclusion bodies greatly resemble mitochondria aggregates in the "nebenkern" commonly observed in spermatocytes during early stages of spermatogenesis. The matrix of most of the large inclusion bodies was finely filamentous to finely granular. In general the periphery of the body was denser and more granular than the center (figs. 7, 8, and 11). In most of the large inclusion bodies there were small round particles of density ranging from slightly more than that of the surrounding matrix up to very dense. The particles themselves varied in size and form from several times the diameter of the fine granules of the matrix up to spheres of size and density equivalent to the intranuclear virus particles. Fully formed particles have been seen immediately within the limiting membrane (figs. 6 and 11) and scattered throughout the matrix of the inclusion body (figs. 7, 8, 10, and 11). They are sometimes closely associated with the internal membranes of the inclusion body (figs. 10 and 11). They

may form a crystalline array within a well-defined inclusion body (fig. 12) or noncrystalline aggregates within and immediately outside a poorly defined inclusion body (fig. 11).

The question remains whether the virus particles are formed *de novo* from the filamentous material in the inclusion bodies or whether they result from viral replication in infected and transformed mitochondria. The latter seems more likely in view of the apparent sequence of events in inclusion-body formation. In only 2 instances have single particles been observed in what appear to be normal or early transition stage mitochondria (figs. 7 and 11). The virus particles occurring within the inclusion bodies greatly resemble the intranuclear particles. They have an average diameter of 30 mμ and appear to be composed of balled threads.

Characteristically the subplasma membrane region of the cytoplasm of the tumor cell, for a distance inwards of approximately 150 mμ, is densely filamentous (figs. 15, 17, 19, and 20) and does not possess recognizable RNP granules or other components of normal cytoplasm. Typically, also (figs. 19 through 22, and 24), this area of the cytoplasm contains 2 sets of oriented threads of dense material. Longitudinal cytoplasmic filaments, averaging 70 Å in thickness, parallel the plasma membrane (*see* particularly fig. 20). The filaments are spaced (center to center spacing) 110 Å apart and show an axial period of 70 Å. This period corresponds to the interruptions of the plasma membrane by finer filaments, 25 to 33 Å in thickness, oriented 90° to the membrane, and extending as much as 40 mμ beyond it into the intercellular space. The significance of the modified, filamentous cell border is not yet understood. However, on the basis of the present micrographs and extrapolation from studies on leukemic tissue (to be published), it is most suggestive that both filaments and plasma membrane enter into the formation of the virus particles at the cell border. At least 4 possible stages are represented in figures 17, 19, and 20. The intermediate steps may only be surmised in the present study. In some cortical areas dense accumulations occur in the filamentous area (figs. 19 and 20) suggesting coalescence of filaments. In 2 instances there appear to be cell border projections containing such material. In other instances definitely forming virus particles (figs. 17 and 20) include both plasma membrane and cortical cytoplasm. Finally the matrix of the particles becomes lost with maturation of the intercellular virus.

In several instances large structures composed of granules, filaments, and small vesicles (fig. 18) were observed at the cell border. The membrane-limited vesicles in the subplasmalemmal region were relatively uniform in diameter, averaging 33 to 45 mμ. They are thus similar to the small vesicles included among the intercellular forms.

Intercellular Virus Particles

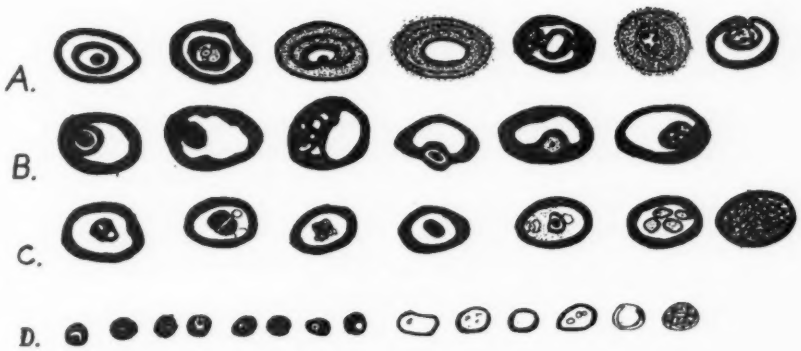
Large numbers of intercellular particles were observed in all of the preparations examined to date. It is perhaps significant that generally

those cells which possess many intranuclear particles show few intracytoplasmic particles. Further, those cells having large numbers of inclusion bodies and intracytoplasmic particles do not show many subplasmalemmal vesicles or virus particles pinching off from the cell border. However, it is possible for one cell to have intranuclear particles, intracytoplasmic inclusion bodies, modified mitochondria, subplasmalemmal cytoplasmic modifications, and intercellular particles still attached to the cell. More frequently one observes simultaneously intranuclear and intercellular or border particles (figs. 13 and 14) but not intracytoplasmic particles in the same area.

The intercellular virus particles were observed most frequently near microvillated borders of the cells (figs. 15, 16, and 20). However, large numbers of particles occurred free in the modified ductules of the salivary-gland tumor and on the stromal side of the nodules of the tumor near and among the collagen fibrils. The intercellular virus particles (figs. 15 through 24) can be grouped into several different types by their appearance. Small particles (D of text-fig. 1) include those of a size (25–35 mμ), form, and density similar to those observed in the nucleus and in cytoplasmic inclusion bodies, and secondly membrane-limited vesicles (33–52 mμ). The latter occasionally contained a small, dense inner mass. Larger virus particles were of the membrane-limited type, similar to those previously described in leukemias and mammary tumors. One of these, type A of text-figure 1, is shown distinctly in figure 18 as a double membrane-limited, oval structure with a central, small, dense nucleoid. The dimensions of this virus are: outer membrane 109 $\times$ 69 mμ, inner membrane 50 $\times$ 39 mμ, and contained-nucleoid 6.6 mμ in diameter. Particles similar to this, or small variations of it, are also seen in figures 17, 19, and 20 through 24. Not always do the membrane-limited viruses contain a nucleoid, perhaps due to plane of sectioning. In the presumably early stages of this form (figs. 17, 19, and 20) the outer, incomplete membranes may appear as up to 4 concentric dense lines. Apparently these coalesce coincident with the loss of matrix to give the definitive intercellular virus form. Just as frequent in occurrence were single membrane-limited particles, roughly spherical to oval, and averaging 103 mμ $\times$ 66 mμ in the oval form and 65 to 70 mμ in the spherical form. These viruses, type C of text-figure 1, contain 1 to 4 dense nucleoidal areas of vesicular or massed thread type, the nucleoid averaging 6 mμ in diameter. Other large, membrane-limited viruses possess a single limiting membrane, are oval in shape, but show an inner membrane-limited aggregate of particles or threads eccentrically placed (type B of text-fig. 1) within the area enclosed by the outer membrane. The dimensions of such viruses are 72 mμ $\times$ 58 mμ outer membrane and 40 mμ $\times$ 30 mμ inner structure.

DISCUSSION

Previous biologic experiments (*4*), fractionation studies (unpublished), and filtration experiments (*2*) have all suggested that the P virus is a



TEXT-FIGURE 1.—Viral forms from the intercellular spaces in the salivary-gland tumor of the mouse as seen in various sectioning planes. Type A forms are apparently related, large, double-membraned viruses usually containing a particle or nucleoidal mass. This type resembles previously described leukemia virus. Type B forms are large, single-membraned viruses with an eccentric nucleoid. Their size and form are similar to those of mammary-tumor viruses. Type C forms are somewhat smaller, single membrane-limited viruses, sometimes filled with a dense mass, sometimes containing one or more inner particles. The nature of this group is undetermined. Type D includes 2 forms—for convenience only. The dense particles are of a size and structure similar to the intranuclear polyoma particles. The vesicular forms of about 40 mμ are not yet specifically identified as viruses.

small particle about 30 mμ in diameter, and that the L virus with which it is associated is probably a large virus approximating 80 mμ in diameter. Intranuclear particles have been reported from 2 tissue cultures infected with P virus. One from the embryonal culture showed nonmembrane-limited particles ranging from 26.5 to 32.2 mμ, with an average of 30 mμ (*7*). The others from murine lymphoma strain P388 D_1 inoculated with the polyoma virus were 27 mμ, spherical, and with no internal structure (*6*). In addition, both from mouse embryonal cultured cells and in the spleen of infected mice, particles were 27 mμ, of low electron density, sometimes showed a limiting membrane, and contained a particle of 4.5 mμ (*5*). Intracytoplasmic particles believed to be P virus seemed to be structurally and dimensionally similar to the nuclear particles (*6*). Intercellular particles have also been reported as similar to the nuclear particles (*5*, *6*). Intracytoplasmic vesicles of 40 to 60 mμ were abundant in mouse embryonal cultured cells infected with polyoma virus (*7*); the relation to the virus infection was undetermined. Viruses 82 mμ in diameter, however, having 2 limiting membranes and an enclosed particle have been observed in the cytoplasm and intercellular spaces of a salivary-gland tumor of the mouse infected with the leukemia agent (*10*).

The present study differs from previous ones in that it was an electron microscopic survey of the tumor itself, and in that several different types of particles were seen within a given cell or within a given tumor nodule area. The intranuclear particles, the particles within the cytoplasmic inclusion bodies, and some of the interstitial particles were apparently

similar to those previously reported as polyoma virus, *i.e.*, they averaged 29.6 mμ, and comprised a dense mass with no surrounding membrane. Crystalline arrays of the particles were seen only in cytoplasmic inclusion bodies. The intranuclear particles appeared to be closely associated with fine filaments which in turn were associated with or continuous with filaments of the nucleolus. It is pertinent to this observation that intranuclear particles in poliovirus-infected tissue-culture cells are also associated with filaments of the nuclear matrix (*12*). High magnifications of the intranuclear particles presently observed show that the particles appear as if formed by a ball of fine threads. Such a possibility in viral formation has been recently suggested (*7*, *13*).

The appearance of P virus particles in inclusion bodies which are most likely modified mitochondria is reminiscent of previous observations of the formation of chicken myeloblastosis particles (*14*). The relationship of the inclusion body particles to the nuclear particles can only be surmised.

The modification of the peripheral cytoplasm and the presence of threads of 2 sizes and orientations suggest that virus particles may be formed *de novo* from the threadlike material in this area of the cytoplasm. The formation of the outer virus membrane from evaginated plasma membrane has been previously observed in mammary-tumor viruses (*15*, *16*), herpes simplex (*17*), and the Friend leukemia virus (*18*). It would appear from the present study that the viral formation and release involve not only modified plasma membrane but also cortical cytoplasmic material.

The observed intercellular viruses assume several different forms. The form could be related to the method of release of the virus, to the cyclic stage, or to the virus species. The small 30 mμ interstitial particles appear similar to those reported previously as polyoma virus. The small vesicles have not been observed previously. The large membrane-limited viruses appear in some respects similar to previously recorded viruses. The double membrane-limited particles, in which the 2 membranes are concentric, are similar to previously reported leukemia viruses (*10*, *19*). The membrane-limited virus of like outer diameter but with an eccentric inner membrane-limited particle appears in many respects to be similar in form to, although smaller than, the mammary-tumor agent (*14*). The presence of multiple mammary tumors would perhaps account for these forms. The particle or virus with a single outer membrane and containing several small vesicles or dense particles has not been observed previously.

Thus we are faced with variation in form, such as that suggested in earlier studies on leukemia viruses (*10*, *18*), or with the presence of several different types of viruses within a given tumor. Although at present the identity of the several different types of viruses cannot be given with any assurance, it seems most likely that the dense intranuclear particles and interstitial particles of similar size and density are the P viruses. It seems also likely that at least 2 viruses are present in the cytoplasm of these tumor cells; one in the inclusion bodies, a second type formed at the cell periphery. Some of the variations in form noted in the interstitial viruses

could be related to the mode of viral release, *i.e.*, in reverse pinocytosis all structures within a given cytoplasmic area are enclosed within a pinocytotic vesicle forming in that area. It is also logical to assume that some of these viruses could be contaminants and that one should not expect to find only the P virus in the salivary-gland tumor from these mice. On the basis of the close association of the P virus and the L virus, it is logical to expect at least 2 viral forms in the tumor, even though the 2 agents, when injected separately, cause dissimilar reactions in the animals.

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All figures are tumor cells.

PLATE 2

FIGURE 1.—General view through 2 cells of salivary-gland tumor of mouse. Nuclei (nu) occupy major portion of cells; cytoplasm (cy) is extremely small. Nucleoli (ncl) are prominent and diffuse. *Lower* nucleus contains patches of viral particles (v). Mitochondria (mi), Golgi complex (g), many RNP granules and few vesicles of endoplasmic reticulum feature these cells. Cell borders irregular, leaving wide intercellular spaces. × 32,500

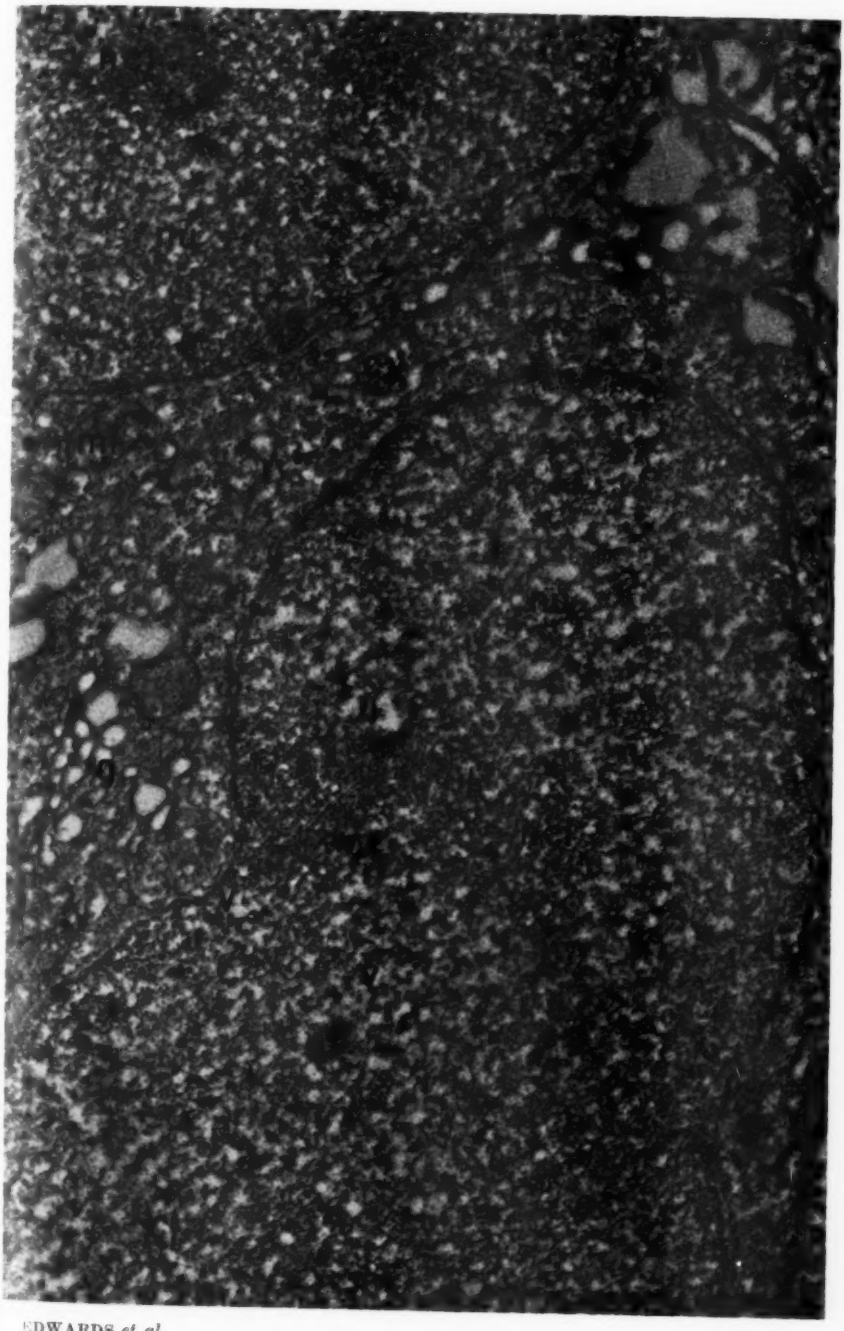

PLATE 3

FIGURE 2.—Detail of nucleus (nu) with small patch of viruses (v). Adjoining cytoplasm contains mitochondria (mi) and inclusion body (ib). × 60,000

FIGURE 3.—In several instances, as in this figure, entire nuclear area was occupied by virus particles (v) of uniform size but varying densities and apparent (at this magnification) varying structure. × 60,000

FIGURE 4.—Highly magnified portion of particles in figure 3. Particles would seem to be masses of threads, as seen in a partially raveled particle (*single arrow*), and regularly wound threads seen in cross and longitudinal section (*double arrows*). Two particles seem to be interconnected by parallel rows of threads (*triple arrows*). × 600,000

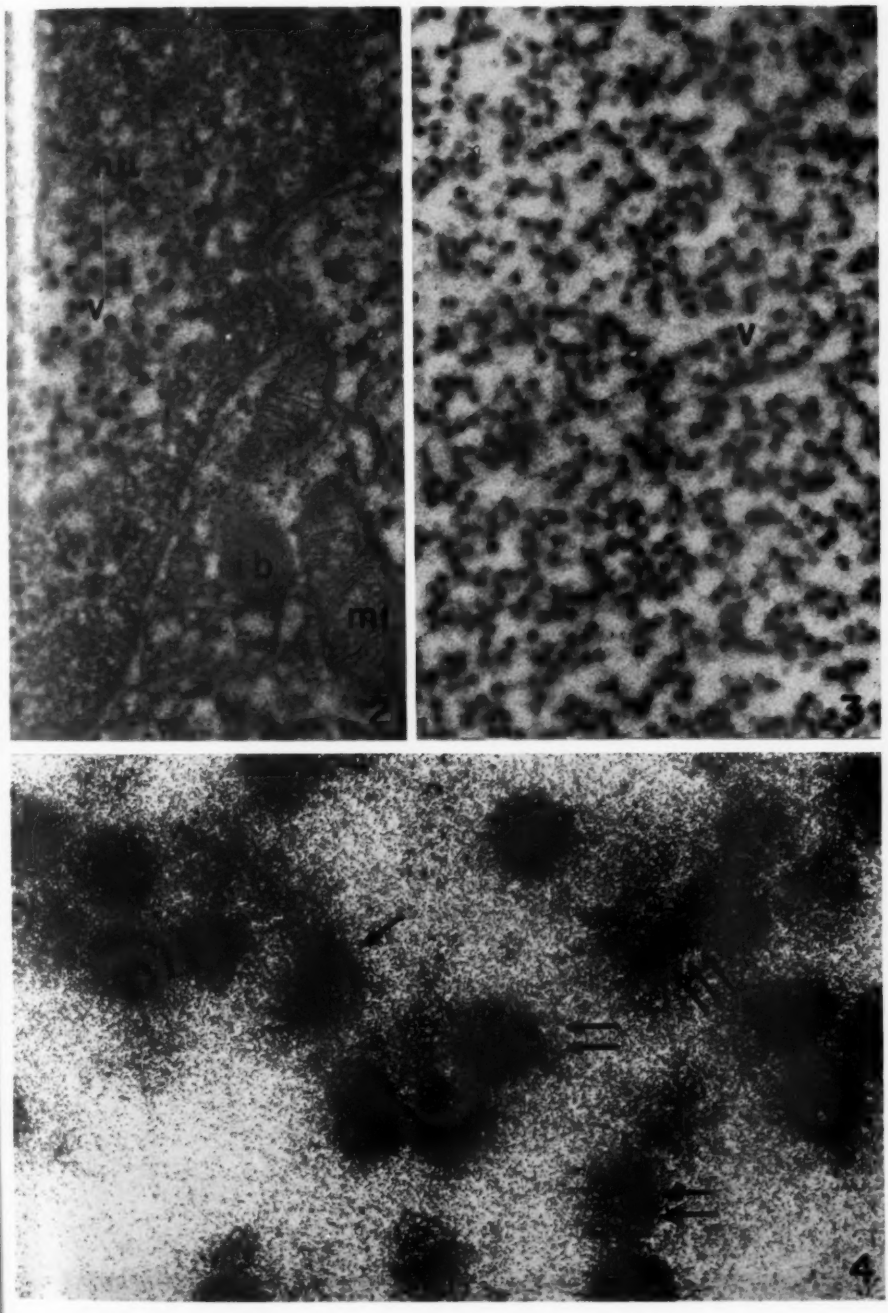
nu
v
b
mi
2
v
3
4

PLATE 4

FIGURE 5.—Portion of nucleus (nu) showing relation of nucleolus (ncl) to fine filaments (f) with which virus particles (v) are associated. × 60,000

FIGURE 6.—Detail of portion of cytoplasm showing membranes of endoplasmic reticulum (er), mitochondria (mi), and 2 inclusion bodies. In *upper* body (ib_2), matrix is finely granular. In *lower* body (ib_1), matrix is granular and filamentous and developing virus particles (v) occur along periphery. × 60,000

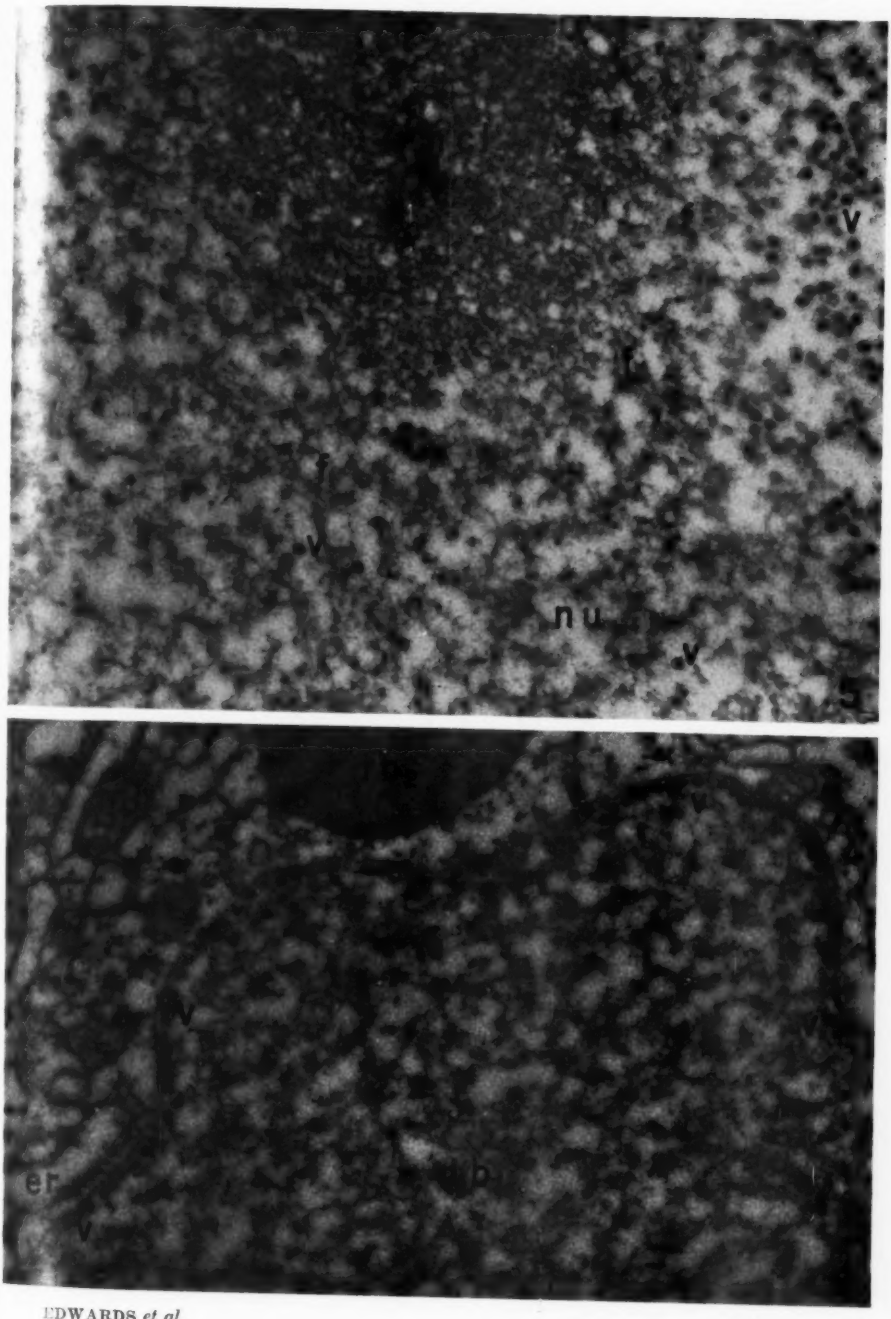
V
f
V
nu
V
V
V
er
V
b

PLATE 5

FIGURE 7.—Cytoplasmic area of tumor cell showing apparently normal mitochondrion (mi_1), mitochondrion with dense outer membrane and diffuse inner membranes (mi_2), and mitochondrion limited by dense membranes and with a dense matrix but no inner membranes (mi_3). Inclusion bodies (ib_1, ib_2, and ib_3) have dense matrices in which virus particles are developing. × 60,000

FIGURE 8.—Cytoplasm showing normal mitochondria (mi) and inclusion bodies (ib_1, ib_2) in one of which developing particles may be seen (ib_2). × 60,000

FIGURE 9.—Detail of cytoplasmic area of tumor cell showing membranes of endoplasmic reticulum (er) and inclusion body (ib) in which vestigial inner membranes and membrane-enclosed structure may be seen. × 60,000

FIGURE 10.—Detail of inclusion body (ib) in cytoplasm in which vestigial membranes (m) and developing virus particles (v) appear. × 60,000

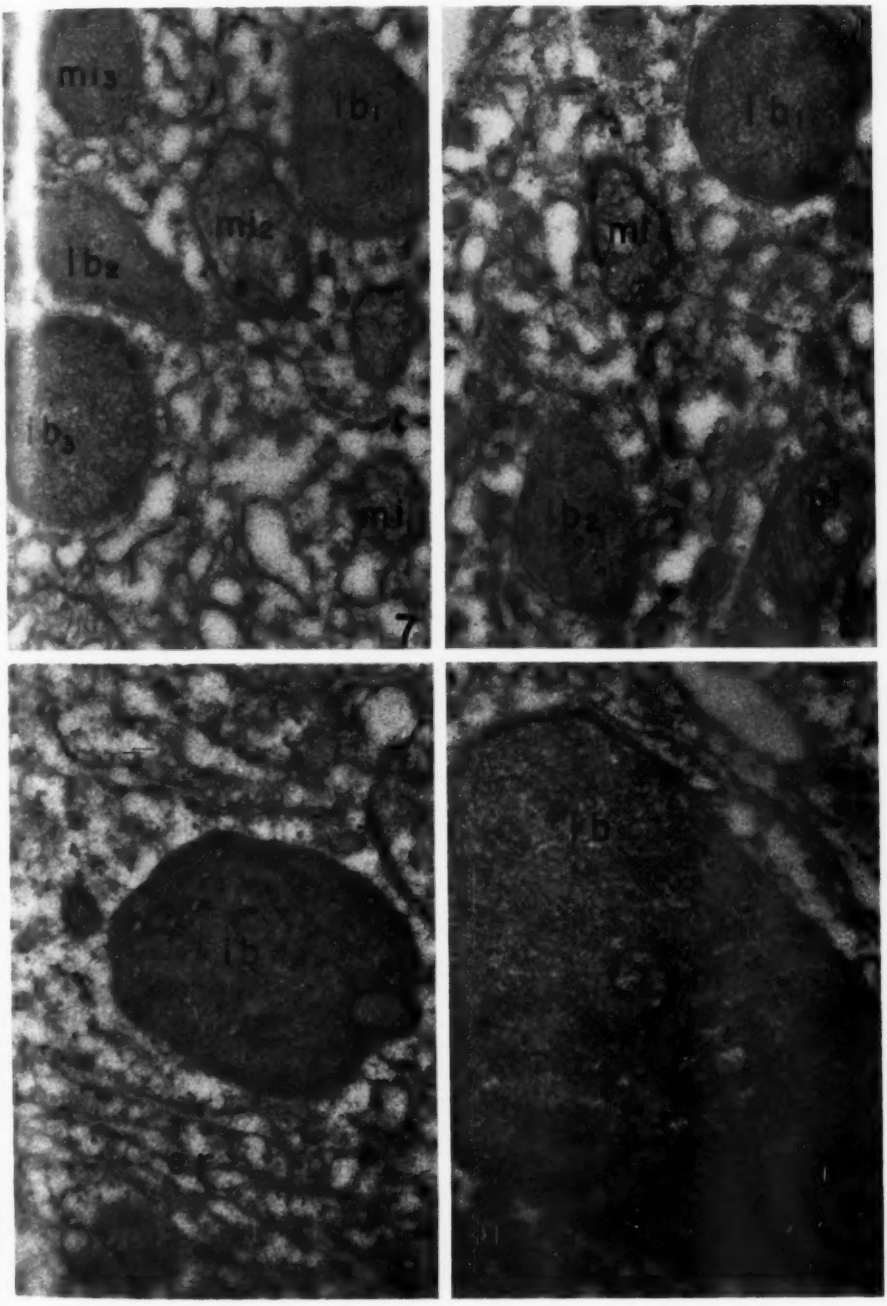

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PLATE 6

FIGURE 11.—Portion of cytoplasm. Mitochondria appear normal (mi), or with densely outlined limiting membranes (mi_2), and with dense matrix but no cristae or with the double membrane outlined (mi_3) and faint matrix. Inclusion bodies appear as only double membrane (ib_1) within and without which are seen virus particles (v), as membrane-limited body containing inner membrane-limited bodies (ib_2), as a membrane-limited body showing inner granular to filamentous matrix and peripheral matrix of particles and vesicles (ib_3), and as structure in which numerous developing particles are visible (ib_4). The rest of cytoplasm shows RNA granules and endoplasmic reticulum (er). × 60,000

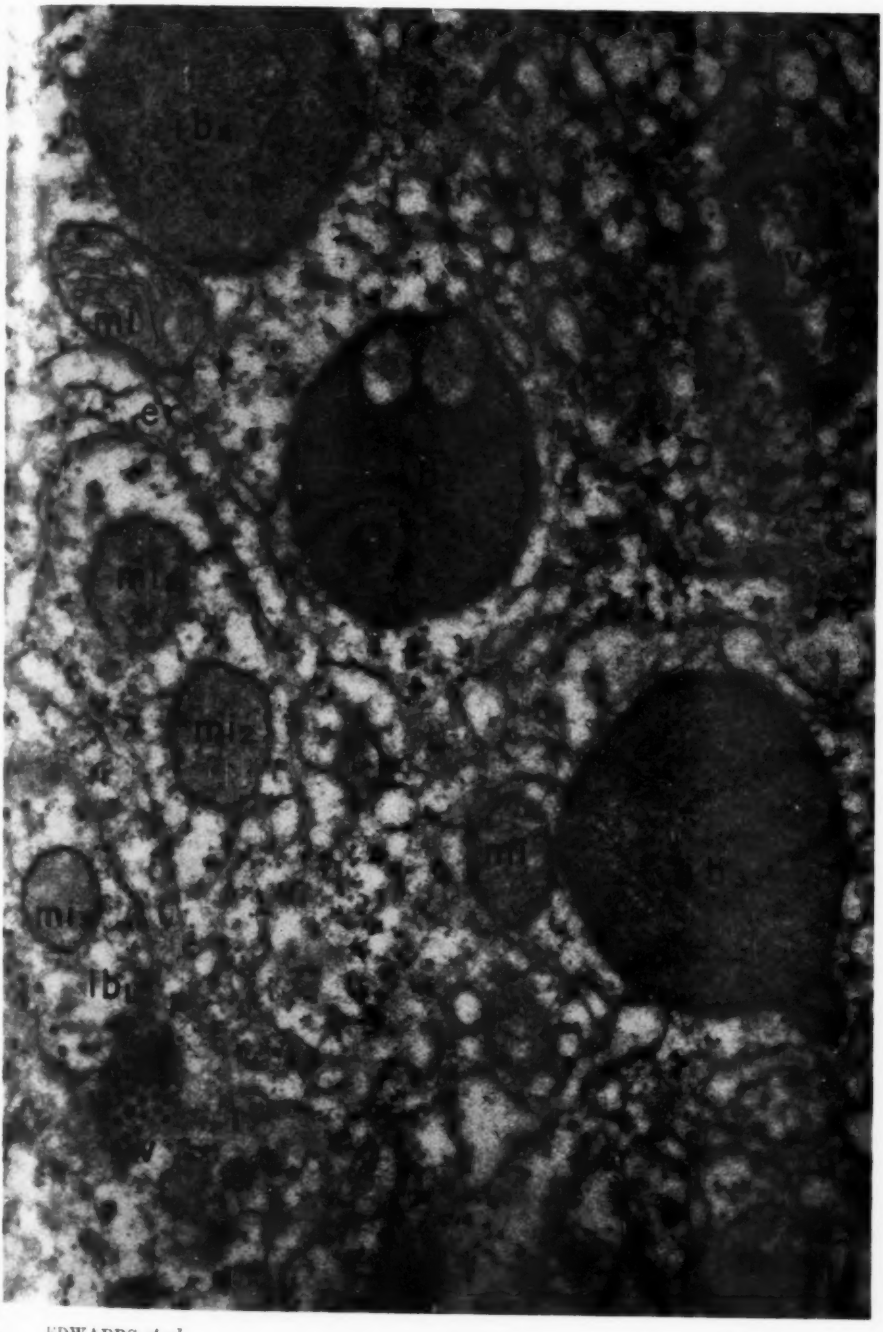

mi
er
mi
mi
mi
mi
lb
v

PLATE 7

FIGURE 12.—Cytoplasm of parotid-tumor cell showing normal mitochondria (mi) and inclusion body (ib) containing crystalline array of virus particles (v). × 60,000

FIGURE 13.—Section through periphery of 2 cells. Nucleus (nu) of cell on *left* contains scattered virus particles (v_1); cytoplasm contains densely outlined mitochondrion (mi) and vesicles in modified peripheral cytoplasm. Interstitial virus particle (v_2) is double membrane limited. × 60,000

FIGURE 14.—Section through cell and adjoining intercellular space showing virus particles (v_1) in nucleus (nu), modified peripheral cytoplasm (cy), and double membrane-limited virus particles (v_2). × 60,000

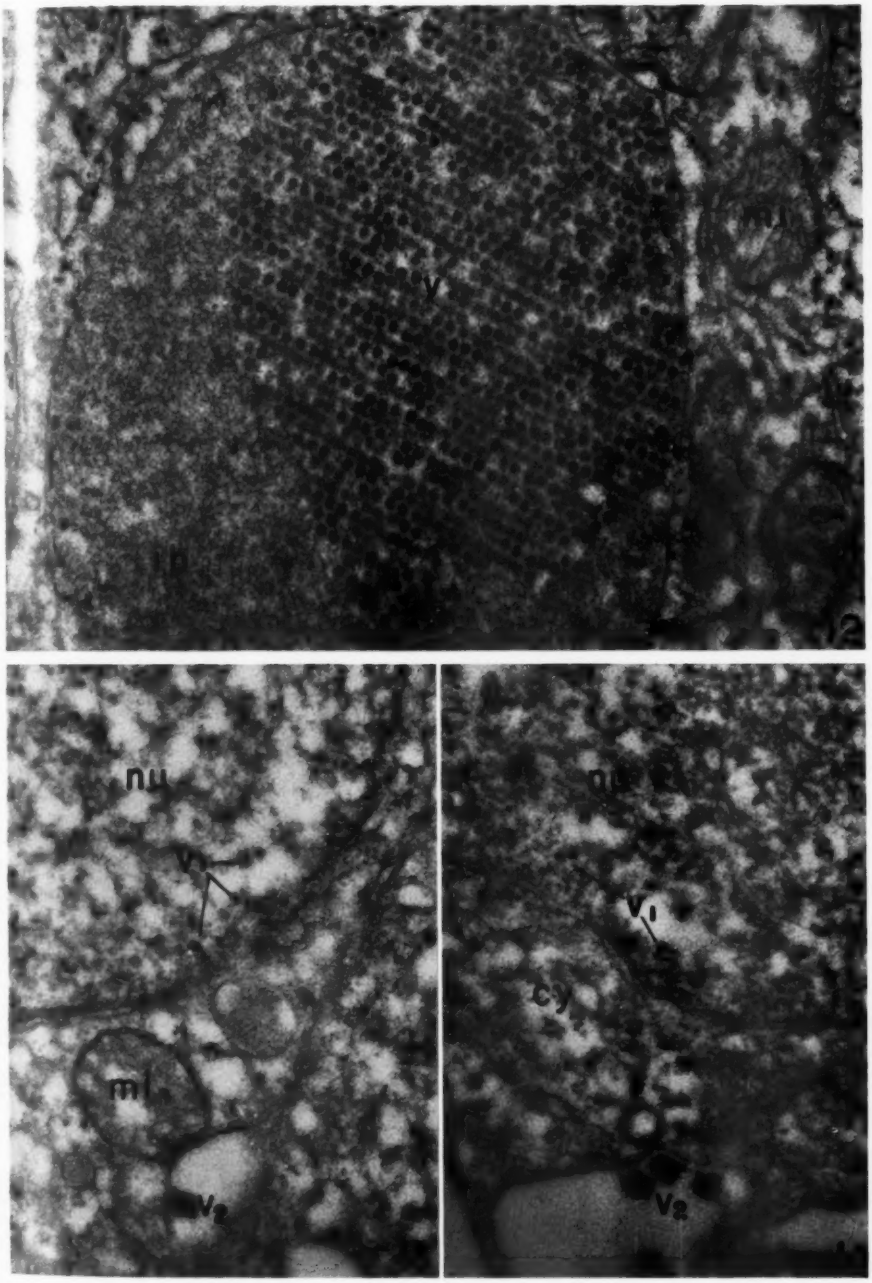

V
nu
V1
V2
cy

PLATE 8

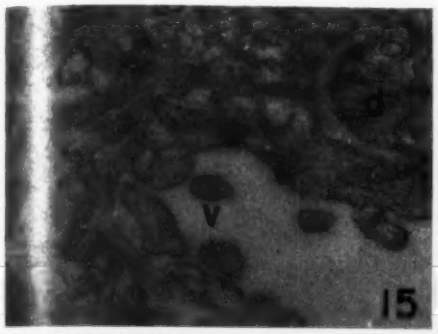
FIGURE 15.—Double membrane-limited virus (v) in intercellular space. Two cells *above* abut by thick-type desmosome (d). × 60,000

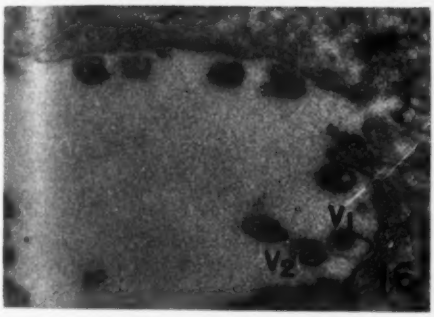
FIGURE 16.—Viral types at cell borders. At *upper left* and *lower right* are double membrane-limited forms (v_2). At *right* is single outer membrane with eccentric membrane-limited nucleoid types (v_1). *Note* fine filamentous component of cytoplasmic periphery. × 60,000

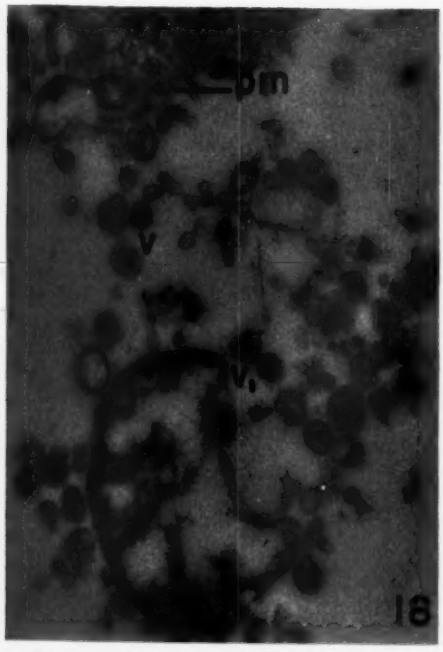
FIGURE 17.—Probable stages of virus formation at cell border. At v both thick and fine filaments of cytoplasmic (cy) periphery and plasma membrane form a small intercellular projection. At v_1 the projection appears as almost complete virus. Matrix of virus is continuous with cytoplasmic matrix. Several dense lines representing viral coats are continuous with plasma membrane and thick cortical cytoplasmic filaments. At v_2 free virus shows complete inner membrane but dense matrix and several incomplete outer membranes. At v_3 inner and outer membranes are distinct but matrix is still present between them. × 100,000

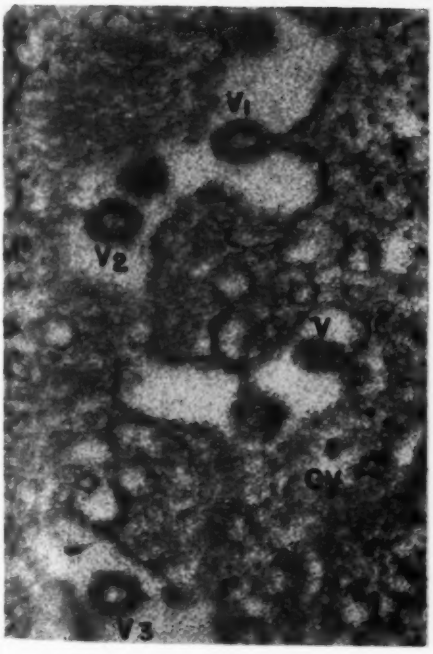
FIGURE 18.—Detail of cell border and adjacent intercellular space. In cytoplasm (cy) and at plasma membrane (pm) may be seen both small vesicles and large membrane-enclosed vesicles. In intercellular space occur dense double membrane-limited viruses (v), single membrane-limited particles (v_1), and small vesicles and particles (v_2). × 60,000

FIGURE 19.—Detail of intercellular viruses (v) and adjacent cell border. Subplasma membrane cytoplasm (cy) consists of or contains longitudinal thick threads (th) and fine threads at right angles to them. At several points along border occur projections representative of possible stages of virus formation. × 120,000


V
15


V1
V2
16


pm
V
V1
18


V1
V2
V
CV
V3

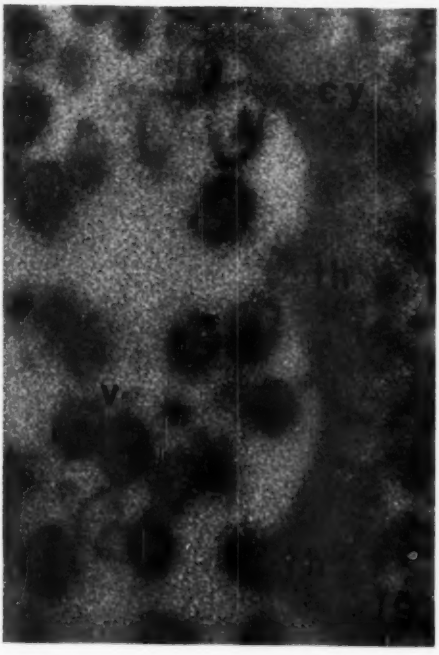
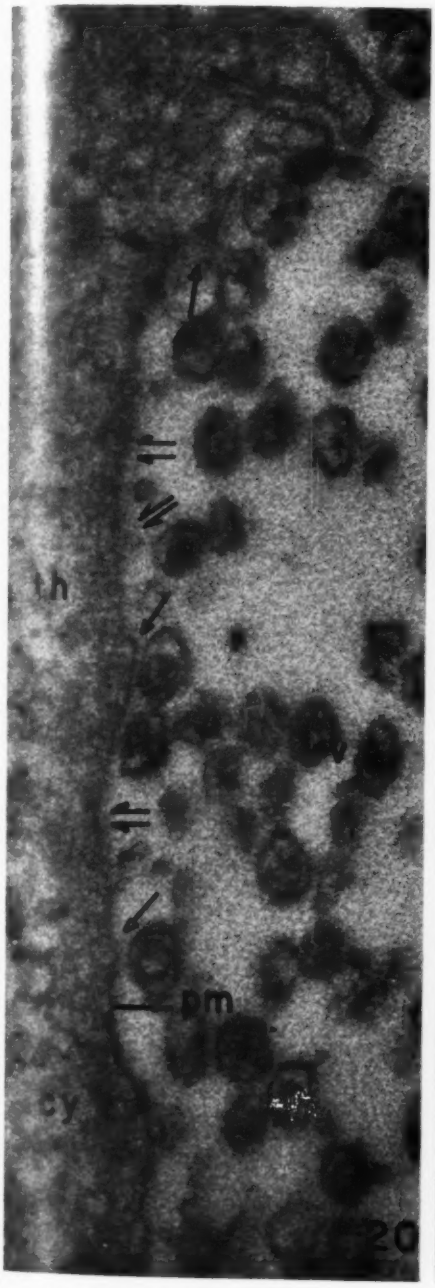

CV
V

PLATE 9

FIGURE 20.—Detail of tumor-cell border and adjacent intercellular space. Peripheral cytoplasm (cy) contains dense, parallel, longitudinal threads (th) and finer threads at 90° to them, the latter extending through modified plasma membrane (pm) into intercellular space (*double arrows*). Where an incomplete virus occurs at the border (*upper single arrow*) both thick and fine threads may be seen within it. Where virus is formed and free of the cell (*center and lower single arrows*) fine threads may still attach to both cell and virus. × 120,000

FIGURE 21.—Cell border (cy) and adjacent intercellular space in which all the viral forms (v) schematized in the text-figure may be seen. × 60,000


h
pm
cy
20

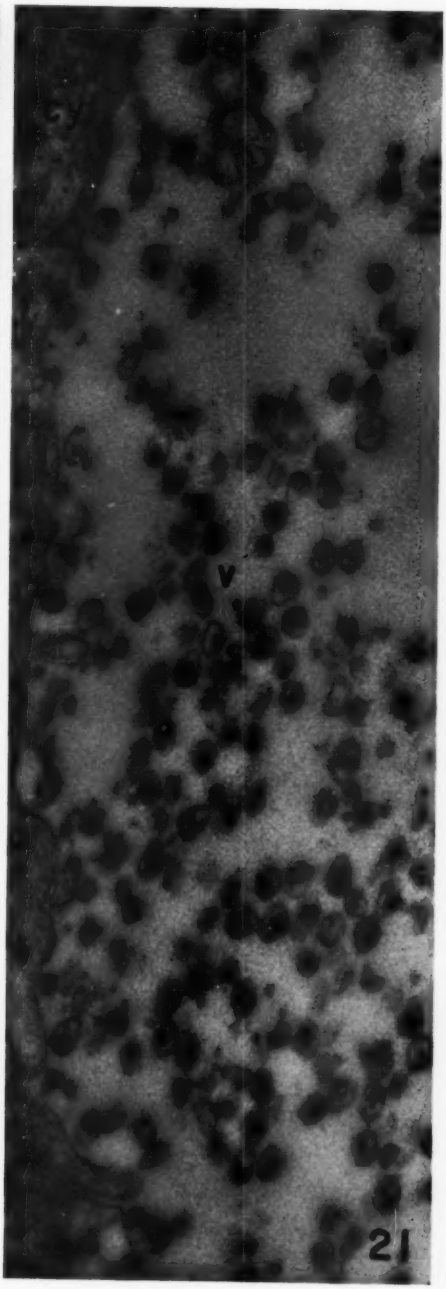
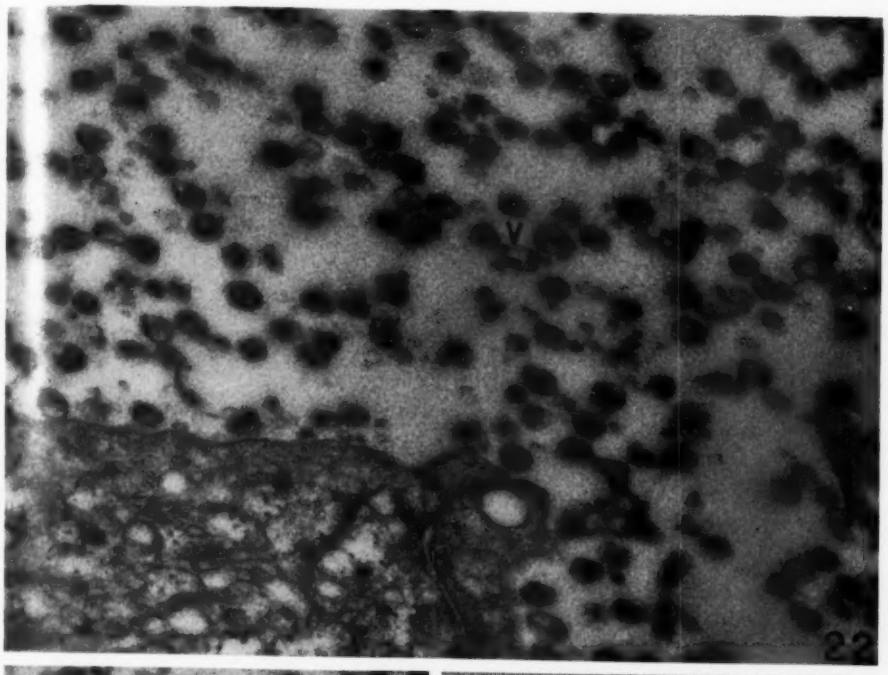
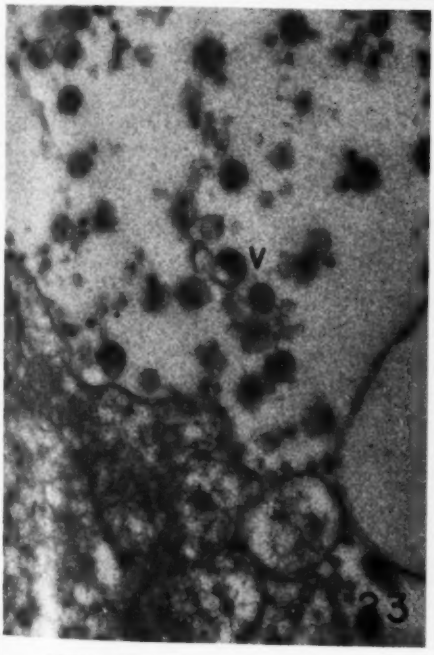
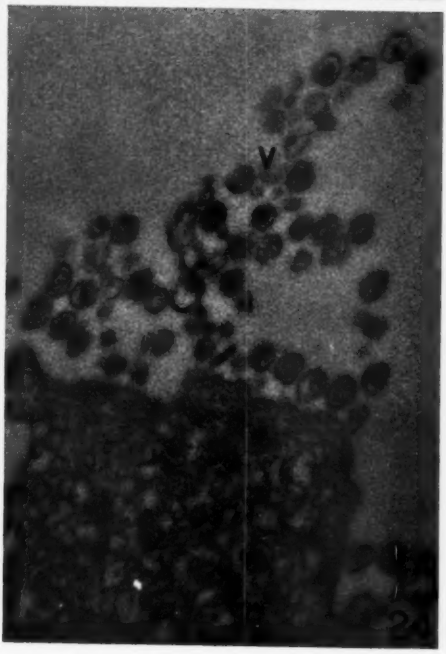

v
21

PLATE 10

FIGURES 22, 23, and 24.—Cell peripheries and intercellular spaces showing several types of virus particles (v). × 60,000


V
22


V
23


V
24

The Carcinogenic Nitrogen Heterocyclics in Cigarette-Smoke Condensate [1,2]

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SUMMARY

The identification of 3 nitrogen heterocyclic carcinogens in cigarette tar is described. These compounds, 7*H*-dibenzo[*c*,*g*]carbazole, dibenz[*a*,*j*]acridine, and dibenz[*a*,*h*]acridine, were present in cigarette tar in concentrations of 0.07γ, 0.27γ, and 0.01γ per 100 cigarettes, respectively. The compounds were identified by their spectra and R_F values on paper chromatograms. It was also shown that dibenz[*a*,*j*]-acridine and dibenz[*a*,*h*]acridine were formed in the pyrolysis of nicotine and of pyridine at 750° C.—J. Nat. Cancer Inst. 25: 53–61, 1960.

THE CHEMICAL constitution of cigarette tar has been examined in this laboratory in an attempt to account for its observed carcinogenic activity to mouse skin. In the course of this work a study was carried out on the polynuclear aromatic hydrocarbons in cigarette tars (*1–3*). Seven carcinogenic compounds were among the 19 higher aromatic hydrocarbons identified in our work. The concentrations in which these known carcinogens were present were insufficient to account for the observed biological activity (*4*, *5*). Further studies were therefore undertaken to determine the nature of some of the other biologically active components in cigarette tar.

In this communication we wish to report the identification of dibenz-[*a*,*h*]acridine (I), dibenz[*a*,*j*]acridine (II), and 7*H*-dibenzo[*c*,*g*]carbazole (III) in cigarette tar in concentrations of 0.01γ, 0.27γ, and 0.07γ per 100 cigarettes, respectively. All 3 compounds obtained in this work have given positive results in carcinogenicity experiments (*6*). These compounds have not been reported by previous investigators (*7*).

Experimental evidence suggests that the dibenzacridines are formed

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[3] The authors are indebted to Dr. Norton Nelson for his interest and encouragement in this work.

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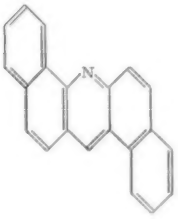
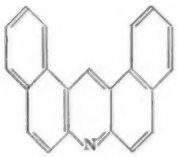
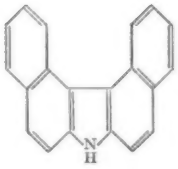
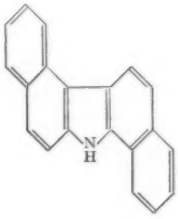
in the burning cigarette by the pyrolysis of pyridine, nicotine, and other substituted pyridine bases.

I 

 II

III 

 IV

EXPERIMENTAL

Synthesis of Authentic Compounds

Dibenz[*a,h*]acridine, I: Condensation of α-naphthylamine, β-naphthol, and paraformaldehyde by the previously described procedure (*8*) yielded dibenz[*a,h*]acridine, m.p. 228° C., reported (*8*) m.p. 228° C. The ultraviolet absorption (*9*) and fluorescence emission (*10*) spectra agreed with the published spectra.

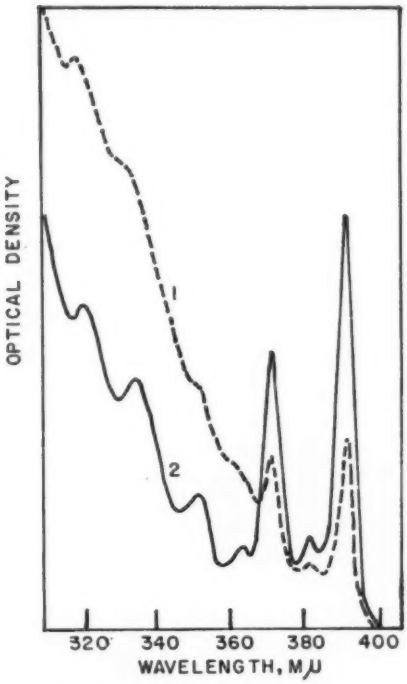
Dibenz[*a,j*]acridine, II: Condensation of β-naphthylamine, β-naphthol, and paraformaldehyde by the previously described procedure (*8*) yielded Morgan's base (*11*), which is a molecular complex consisting of dibenz[*a,j*]-acridine and 7,14-dihydrodibenz[*a,j*]acridine (*11*), m.p. 234 to 235° C. d. [reported (*11*) m.p. 232–240° C., and 246–248° C.]. Morgan's base was converted to dibenz[*a,j*]acridine by refluxing in acetic acid (*11*), m.p. 220° C. [reported m.p. 216° C. (*8*), 219–221° C. (*11*)]. The ultraviolet absorption and fluorescence emission (*10*) spectra agreed with the published spectra.

7*H*-Dibenzo[*c,g*]carbazole, III, and 7*H*-dibenzo[*a,g*]carbazole, IV: Buu-Hoï's procedure (*12*) was used to synthesize these compounds. The ultraviolet absorption spectra (*13*) agreed with the published spectra. 7*H*-Dibenzo[*c,g*]carbazole, dissolved in cyclohexane, showed fluorescence excitation maxima at 280, 305, 335, 350, and 365 mμ and fluorescence emission maxima at 363, 382, 400, and 424 mμ. 7*H*-dibenzo[*a,g*]carbazole

showed fluorescence excitation maxima at 287, 298, 337, 345, and 361 mμ and fluorescence emission maxima at 367, 386, 408, and 430 mμ.

Identification of the Dibenzacridines in the Basic Fraction of Cigarette Tar

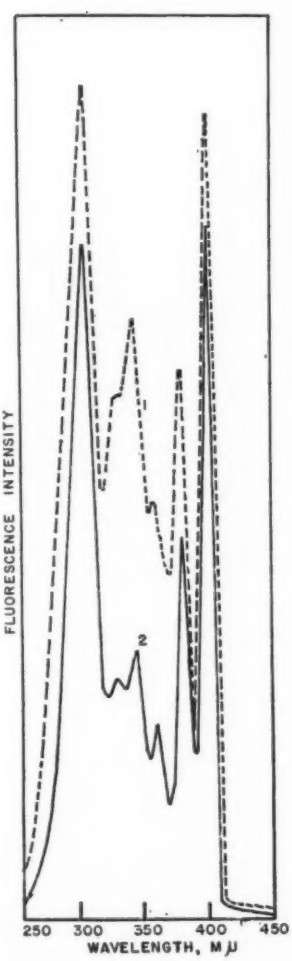
Whole tar (*14*), 250 gm., was dissolved in 250 ml. of benzene and extracted with 1 l. of 10 percent aqueous sulfuric acid in small portions. The acidic extract was washed with benzene, neutralized with anhydrous potassium carbonate, and more potassium carbonate was added until the basic fraction separated out as an oil. The bases were then extracted from the aqueous mixture with benzene and dried, and the benzene was distilled off in a nitrogen atmosphere. The residue was distilled at 100° C. and 0.5 mm. to yield 27 gm. of a colorless distillate containing nicotine, quinoline, pyridine, and related basic compounds. The residue 10 gm. (*i.e.*, the basic fraction constituted 15% of whole tar) was chromatographed on 250 gm. of Alcoa activated alumina (grade F-20) which had been freshly reactivated at 210° C. for 16 hours. Three fractions were collected: (1) benzene-petroleum ether (b.p. 30–60° C.) 1 : 1; (2) benzene; (3) methanol. Preliminary experiments showed that the dibenzacridines had been eluted in the benzene fraction under these conditions. The benzene eluate, 6 l., was distilled to a small volume at 50° C. and 20 mm. in a nitrogen atmosphere. The residue was chromatographed first on Whatman #1 chromatographic paper (*2*), then on S. & S. acetate paper, grade 2043b (*3*), and again on Whatman #1 paper. The areas corresponding in R_F to the 2 dibenzacridines (I and II) were eluted each time and rechromatographed. Fluorescence and ultraviolet absorption spectra were taken of the final fractions. The fluorescence spectra for both fractions were identical with the spectra of the authentic compounds. There was background absorption in the ultraviolet spectra but bands in the 320 to 400 mμ region did appear and agreed in their positions with the ultraviolet absorption maxima of the authentic compounds. The ultraviolet absorption spectrum of dibenz[*a*,*j*]acridine and the corresponding cigarette-tar fraction are shown in text-figure 1. The fluorescence excitation spectrum of a fluorescent compound shows maxima at the same wavelengths as the ultraviolet absorption maxima. Acceptable fluorescence excitation spectra were obtained for all the cigarette-tar fractions. Background absorption in the ultraviolet spectra therefore did not hinder clear-cut identification of the dibenzacridines. The fluorescence spectra for dibenz[*a*,*j*]acridine and the cigarette-tar fraction of the same R_F are shown in text-figures 2 and 3. An additional aid in the identification of the dibenzacridines was the fluorescence of these compounds in concentrated sulfuric acid. Both compounds showed a shift to longer wavelengths in their excitation and emission spectra when dissolved in sulfuric acid as compared to their fluorescence spectra when dissolved in cyclohexane. Thus dibenz[*a*,*j*]acridine in sulfuric acid showed excitation maxima at 300, 425, and 445 mμ and emission maxima at 452 and 478 mμ. The shift is even larger for dibenz[*a*,*h*]acridine in sulfuric acid. This



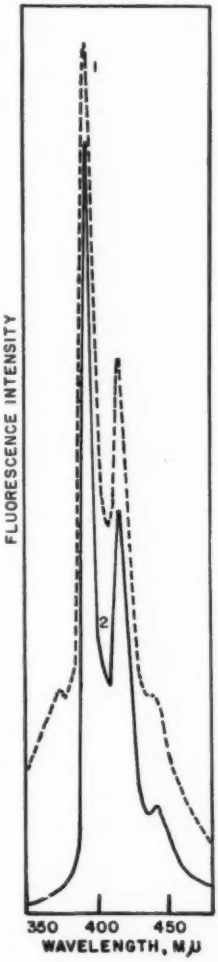
TEXT-FIGURE 1.—Ultraviolet absorption spectra: (1) Unknown; (2) dibenz[*a,j*]acridine, solvent cyclohexane.

compound, dissolved in sulfuric acid, showed excitation maxima at 310, 400, 430, 442, and 457 mμ and emission maxima at 460, 485, and 500 mμ. The dibenzacridines isolated from cigarette tar when dissolved in sulfuric acid showed fluorescence spectra which agreed well with those of the authentic compounds in the same medium. As an example, the fluorescence spectra of dibenz[*a,j*]acridine and the corresponding cigarette-tar fraction in sulfuric acid solution are shown in text-figures 4 and 5.

The concentrations of the dibenzacridines given in table 2 are average values obtained from the ultraviolet and fluorescence spectra. The ultraviolet spectra gave values that were approximately 10 percent higher than the values obtained from the fluorescence spectra. Quenching of fluorescence by traces of impurities is probably responsible for the lower values obtained from the fluorescence spectra. The shortest wavelength emission band in the fluorescence spectra and the longest wavelength absorption band in the ultraviolet spectra were used to calculate the concentrations by comparing the peak heights of these bands with the same bands obtained from solutions of similar, but known, concentrations of the authentic compounds. For the quantitative analyses by fluorescence, very dilute solutions were used to eliminate concentration quenching.



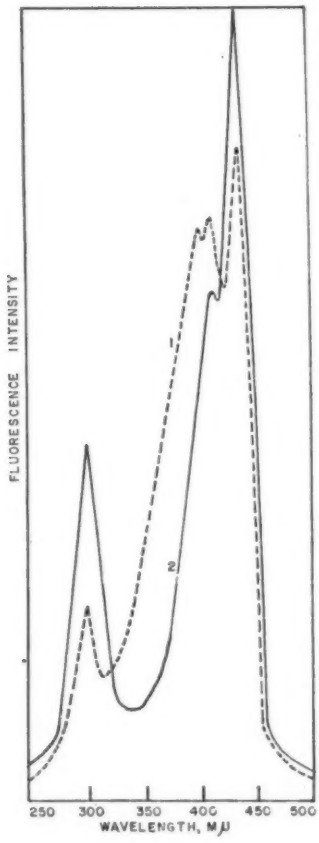
TEXT-FIGURE 2.—Fluorescence excitation spectra, emission at 400 mμ: (1) Unknown; (2) dibenz[*a,j*]acridine, II, solvent cyclohexane.



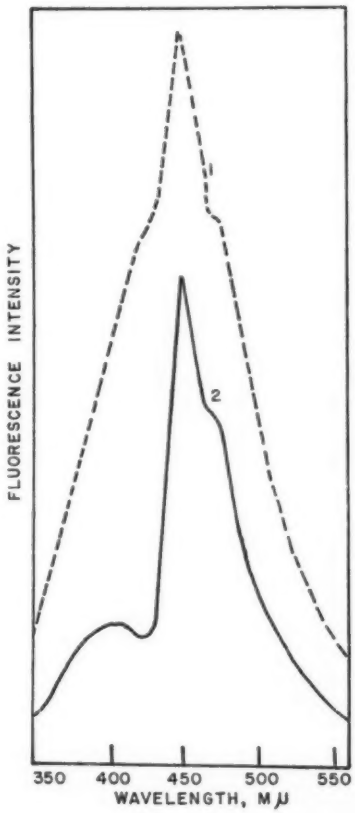
TEXT-FIGURE 3.—Fluorescence emission spectra, excitation at 300 mμ: (1) Unknown; (2) dibenz[*a,j*]acridine, II, solvent cyclohexane.

Identification of 7*H*-Dibenzo[*c,g*]carbazole in the Neutral Fraction of Cigarette Tar

The neutral fraction of cigarette tar, which comprises 19 percent of whole tar, was prepared as described previously (*2*) and chromatographed on activated alumina. The benzene eluate from this chromatogram was evaporated to dryness and rechromatographed on alumina. The petroleum ether- (b.p. 30–60° C.) ether (1:1) eluate from the second chro-



TEXT-FIGURE 4.—Fluorescence excitation spectra, emission at 460 mμ: (1) Unknown; (2) dibenz[*a*,*j*]acridine, II, solvent concentrated sulfuric acid.



TEXT-FIGURE 5.—Fluorescence emission spectra, excitation at 320 mμ: (1) Unknown; (2) dibenz[*a*,*j*]acridine, II, solvent concentrated sulfuric acid.

matogram was chromatographed repeatedly on Whatman #1 paper (*2*). 7*H*-Dibenzo[*c*,*g*]carbazole was identified with the authentic compound by its R_F, fluorescence excitation, and emission spectra. There was background absorption in the ultraviolet spectrum but bands in the 320 to 400 mμ region did appear and agreed with the ultraviolet maxima of the authentic compound. 7*H*-Dibenzo[*a*,*g*]carbazole could not be found in the neutral fraction.

The R_F values of the authentic compounds used in this study are shown in table 1.

Pyrolysis of Pyridine and Nicotine

Pyridine and nicotine were each pyrolysed at 750° C., in a vycor tube (*i.e.*, approximating the temperature of a burning cigarette). A stream

TABLE 1.—R_F values of authentic compounds

Compound	R_F values	
	Whatman #1 paper; solvent, *N,N*-dimethylformamide-hexane (*2*)	Acetate paper; solvent, toluene-methanol-water; 1:10:2 (*3*)
7*H*-Dibenzo[*a,g*]carbazole	0. 15	0. 18
7*H*-Dibenzo[*c,g*]carbazole	0. 15	0. 30
Dibenz[*a,h*]acridine	0. 61	0. 12
Morgan's base	0. 35	0. 35
Dibenz[*a,j*]acridine	0. 35	0. 35

of nitrogen was passed through the combustion tube at a rate of 3 l. per minute. The highly fluorescent pyrolysates were distilled in vacuum at 100° C. and 20 mm. The residues were chromatographed on alumina followed by paper chromatography, as described for the isolation of the dibenzacridines from cigarette tar. Both dibenz[*a, j*]acridine and dibenz[*a,h*]acridine were obtained; the former was again obtained in much larger quantities than the latter.

DISCUSSION

With this work a total of 10 carcinogenic polynuclear aromatic compounds (hydrocarbons and heterocyclics) in cigarette tar have now been identified in this laboratory. These compounds and their available estimated concentrations are listed in table 2.

The results obtained in the pyridine and nicotine pyrolyses suggest that the dibenzacridines are synthesized in the burning cigarette from pyridine, nicotine, and other substituted pyridine bases. These components are known to constitute between 5 and 15 percent of whole tar.

In view of inevitable losses in the fractionation of cigarette tar, the actual concentrations of the carcinogens in the original tar are undoubtedly higher than the values reported in this work.

Andervont and Shimkin (*15*) compared the carcinogenicity of benzo[*a*]pyrene and several other potent carcinogens including the 3 compounds discussed in this report, using pulmonary-adenoma production in mice as the test system. They compared the relative potencies of 3 compounds with benzo[*a*]pyrene not only for the production of pulmonary adenomas but also for tumor response after skin application and subcutaneous injection, as noted in earlier reports. The different testing procedures gave different relative potencies; thus the 3 heterocyclics here identified were generally more potent than benzo[*a*]pyrene for adenoma induction, but were less active by percutaneous application and subcutaneous injection.

Skin-painting experiments carried out in this laboratory (*16*) on the basic fraction of cigarette tar were negative; however, it is now clear on

TABLE 2.—Carcinogenic aromatic compounds in cigarette-smoke condensate

Compound*	Concentration γ/100 cigarettes
Benzo[*a*]pyrene (3,4-benzpyrene)	0. 50
Benzo[*e*]pyrene (1,2-benzpyrene)	0. 30
Dibenz[*a,j*]acridine (1,2,7,8-dibenzacridine)	0. 27
7H-Dibenzo[*c,g*]carbazole (3,4,5,6-dibenzcarbazole)	0. 07
Chrysene	0. 06
Dibenz[*a,h*]anthracene (1,2,5,6-dibenzanthracene)	0. 05
Dibenz[*a,h*]acridine (1,2,5,6-dibenzacridine)	0. 01
Dibenzo[*a,i*]pyrene (3,4,9,10-dibenzpyrene)	0. 002
Benzo[*c*]phenanthrene	——
Benzo[*j*]fluoranthene (7,8-benzfluoranthene or 10,11-benzfluoranthene)	——

*'*Chemical Abstracts*' nomenclature is used in this paper. Other commonly used names are given in parentheses.

the basis of the data reported here that the concentration of the basic fraction used in those experiments would not have been sufficient to yield skin tumors even if the dibenzacridines had been as potent for the skin as benzo[*a*]pyrene, which is not the case. Wynder (*17*) using more concentrated fractions did report weak tumor activity in the basic fraction.

Accordingly, it is clear that the heterocyclics here described make a significant contribution to the reported carcinogenicity of tobacco tars.

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Rapid Electronic Measurement of Cell Volume and Distribution [1,2]

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SUMMARY

With suitable calibration factors, the Coulter electronic counter may be used for the determination of the number and volume distribution in a variety of cell suspensions; and with additional corrections, the influence of osmotic strength on cell size may be investigated. By this means, the distribution of volume in a variety of tumor-cell populations was studied. Values that agreed satisfactorily with those obtained by other methods were found. The volumes are not distributed normally in such populations, as there is a relative preponderance of larger cells. The logarithm of the volume was normally distributed, however, and the median and standard deviation of this distribution afforded a convenient method of describing such populations.—J. Nat. Cancer Inst. 25: 63–74, 1960.

DURING OUR recent investigation of the reduction of tetrazolium salts by ascites tumor cells, atypical responses were frequently encountered. Although the cell strain appeared uniform in morphological characteristics, from experiment to experiment, quantitative deviations in cell size could not be excluded.

Decreases in the average cell volume during the growth of ascites tumors have been reported by Rice and Shelton for L1210 lymphoid leukemia and Lymphoma #1-c (*1*), by Levy *et al.* for the DBA thymoma (*2*), and by Révész and Klein for the DBA, EL4, and 6C3HED lymphomas (*3*). Klein and Révész (*4*) and Lucké and Berwick (*5*) found no changes in the Ehrlich and MC1M ascites tumors.

Until recently a reliable estimate of average cell volume could be made only by direct measurement of large numbers of cells or by calculations based on the percentage volume occupied by a known number of cells packed by centrifugation. Computation of volume from measurements

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[2] Presented in part at the 50th Annual Meeting, American Association for Cancer Research, April 10, 1959.

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[4] We are grateful to Dr. E. Shelton, National Cancer Institute, National Institutes of Health, for supplying the lymphomas.

[5] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

of diameter is largely restricted to spherical cells and a few other types such as red cells, in which the geometry has been studied in detail (*6*). A correct estimate of volume may be derived from packed-cell volume only under certain conditions, even for red cells (*7*). Values for mean cell volume are erroneously high whenever packing of cells is incomplete. If information concerning the frequency distribution of volume is desired, microscopic measurements of each cell are required.

Accurate enumeration and size distribution of erythrocytes by the Coulter electronic cell counter were reported by Mattern, Brackett, and Olson (*8*) and by Brecher, Schneiderman, and Williams (*9*). Kuchler and Merchant (*10*) applied the technique for counting suspensions of tissue cultures; Kubitschek (*11*) modified and calibrated this instrument for counting and sizing bacteria. Use of this counter to determine accurately the cell-volume distribution of large numbers of cells larger than erythrocytes has not been reported.

The electronic counter was modified and calibrated to permit counting and sizing cells larger than 10 μ in diameter and applied to the determination of cell-volume distribution in several ascites tumor and tissue-culture populations. Because of the large number of cells counted, slight changes in cell size are immediately discernible. This technique is sufficiently sensitive to reveal small changes in cell size as a result of altered osmotic environment or age of population.

MATERIALS

Cell populations.—The cell populations used in this study were: 7 μ puff balls (plant spores),[6] human and chicken red blood cells; the ascites lymphomas, L #1, L1210, and EL4; the Ehrlich E2 clone and Krebs-2 ascites carcinomas, and the MAI strain of HeLa cells.

Preparation of buffer (diluting medium).—A modified Krebs-Ringer phosphate buffer (*12*) was prepared at a 20× concentration and passed through a fine sintered-glass filter to reduce the content of dust particles which increase the background count. Subsequent dilution of the buffer was made with demineralized water. For the osmotic studies, the sodium chloride content was adjusted to the following concentrations: 0.45, 0.6, 0.9, 1.2, and 1.8 percent.

Preparation of cell suspensions

Puff balls (7 μ).—Eight mg. of puff balls was dispersed by slow addition of 1 ml. of absolute ethanol followed by 1 ml. of thymol-saturated 0.9 percent saline. The suspension contained approximately 32×10^6 cells per ml.

Red blood cells.—Human and chicken red blood cells were washed 3 times in buffer containing 5 mg. of human albumin per 100 ml. Dilutions

[6] Stanford Research Institute, Particle Bank, Menlo Park, Calif.

were adjusted to 10^5 cells per ml. Hematocrits were performed in the usual manner (*7*).

Tumor cells.—The ascites tumor cell suspensions were harvested from the host mice into siliconized tubes. Dilutions were made directly from the ascitic fluid into buffer.

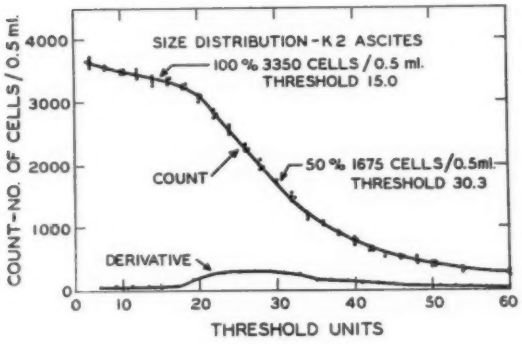
MAI strain of HeLa cells.—HeLa cells were removed from culture tubes by trypsinization and suspended in Eagle's basal medium. Dilutions were made directly into buffer.

Transplantation study.—Twenty-five Swiss BALB/cAnN mice were inoculated with Krebs-2 ascites carcinoma cells (stock tumor) which were serially transplanted every 7 days. Five animals were killed at selected intervals (3, 5, 7, and 12 days) and size distributions made. The tumor cell inoculum was standardized at approximately 20×10^6 cells in 0.2 ml.

INSTRUMENTATION

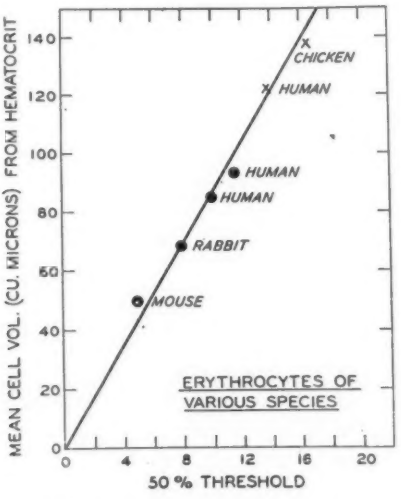
The apparatus [described in detail in (*8*)] consists of 2 parts, the electrode assembly and the required electronic counting circuitry. The platinum electrodes are placed in a buffer solution and an electrical current is made to flow between them. One of the electrodes, however, is immersed in a glass vessel isolated from the other except for a minute aperture (100 μ diameter) through which all the current must flow. With the electrical current on, a suspension of cells is placed in the outer compartment, and a predetermined volume, 0.5 ml., of the suspension drawn through the aperture. If the suspension is sufficiently dilute, most of the cells pass through the hole singly. The passage of each cell through this aperture causes an increase of electrical resistance and results in a voltage pulse proportional to the volume of the cell (*see* later). The instrument counts these pulses as they occur but, in addition, may be adjusted ("threshold") so that only pulses larger than the threshold value are counted. Pulses larger than the threshold value appear brighter on the cathode-ray tube and the lower margin of the bright portion ("brightening line") indicates the threshold voltage. One may determine the distribution of sizes in a population of suspended cells from successive counts at various settings of the threshold control, which selects the sizes of cell to be counted (text-fig. 1).

The threshold control is a linear potentiometer, and under standard conditions these threshold settings are directly related to volume (text-fig. 2). To increase the range of cell volumes which may be measured in this way, the instrument is provided with 2 major adjustments, the aperture current adjustment and the gain control. The aperture current adjustment is a step-type control which limits the amount of current flowing through the aperture; if the current is increased, the height of the voltage pulse is increased. The gain control, also step-type, changes the gain of the amplifier in steps of $\sqrt{2}$; when the gain is increased, the size of



TEXT-FIGURE 1.—Size distribution, Krebs-2 ascites carcinoma. Duplicate counts were made at various threshold settings. Figure illustrates use of derivative curve to obtain the threshold setting corresponding to the smallest members of the population.

the pulse is increased. Except for the possible effect of the increased current on the cells and inconsequential differences in the signal to noise ratio, the aperture current adjustment and the gain control have the same effect, *i.e.*, changing the size of the pulse. By means of these controls, the pulse height from cells of a variety of sizes may be adjusted to fit conveniently in the range of the threshold dial.



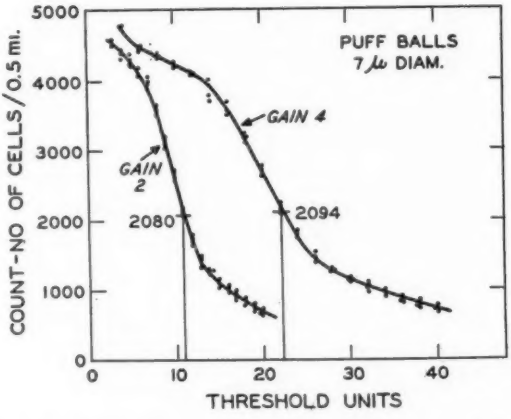
TEXT-FIGURE 2.—The relation between mean cell volume and threshold readings at the 50 percentile point for a variety of erythrocytes. All data collected at the same settings of gain and aperture current.

To make use of this feature of the instrument, the changes in pulse height produced by changes in the gain or aperture current must be accurately known.

Calibration Procedure

Threshold dial zero.—While erythrocytes or puff balls are passing through the aperture, the threshold dial is adjusted so that the threshold voltage is just at zero, as indicated by the coincidence of the brightening line with the base line on the oscilloscope. The knob is reset if necessary so that it reads zero.

Procedure for adjusting threshold zero.—A suspension of approximately 10^4 puff balls per ml. is counted at a series of successive threshold steps to construct a size distribution curve (text-fig. 3). A threshold setting in the steep, most sensitive portion of the curve is selected, a point about 50 percent of total count. Several counts are made at this setting and the threshold and gain settings are then doubled. The count at these new settings should be within 1 or 2 percent of the first count. If not, the zero adjustment is rotated in the direction that reduces differences to about 1 percent. Changes of this internal zero adjustment affect the zero settings of the threshold dial which must be reset until final adjustment is made.



TEXT-FIGURE 3.—Size distribution of 7 μ puff balls at 2 settings of the gain controls. In the example shown, the instrument is considered to be correctly calibrated.

Aperture current calibration.—Each upward step of this adjustment increases the height of the pulse by a factor of approximately two, but this factor varies from step to step and depends on the conductivity of the medium in which the cells are suspended. For solutions having the conductivity of 0.9 percent sodium chloride, the calibration values, supplied by the manufacturer, are listed in table 1.

TABLE 1.—Conversion factors, aperture current *

Aperture current setting used	Aperture current value of calibration: 1	2	3	4	5	6	7	8
1	1.00	1.98	3.92	7.65	14.6	20.6	45.5	67.8
2	0.505	1.00	1.98	3.86	7.38	13.4	23.0	34.2
3	0.255	0.505	1.00	1.95	3.72	6.76	11.6	17.2
4	0.131	0.259	0.514	1.00	1.91	3.48	5.95	8.84
5	0.0685	0.135	0.269	0.524	1.00	1.82	3.12	4.64
6	0.0376	0.0746	0.148	0.288	0.550	1.00	1.71	2.55
7	0.0216	0.0435	0.0861	0.168	0.321	0.585	1.00	1.49
8	0.0150	0.0292	0.0581	0.113	0.216	0.392	0.671	1.00

*If measurements are made at an aperture current setting different from that used to calibrate the threshold dial, the observed threshold reading must be corrected. To determine the appropriate factor, see aperture current setting used and under the appropriate value of the calibration aperture current read the factor. This factor is a multiplier.

These calibration values may be confirmed or estimated for a solution of different conductivity by a method analogous to the calibration of the gain scale. The gain and threshold are adjusted as before to provide a count near the 50 percent point. The aperture current is changed and the new threshold required to reproduce the same count is determined. These 2 thresholds will be in the same ratio as the change in pulse height.

Use of the calibration factors.—Cells of known volume are sized at some convenient setting of gain and aperture current, and the factor which converts threshold dial readings to volume is calculated. When a different setting of the gain or aperture current is required for measuring cells of different volume range, appropriate factors must be used to convert the measurements to the standard gain and aperture current (tables 1 and 2). The desired volume is the threshold reading, multiplied by threshold factor and the appropriate corrections. Coincidence correction at the counting levels used (6,000 cells per 0.5 ml. maximum, 140 μ aperture) does not exceed 4 percent and was not applied.

TABLE 2. Conversion factors, amplifier gain*

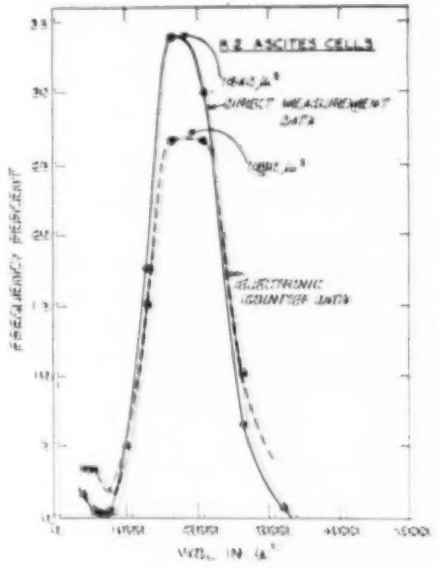
Gain setting used	Gain value of calibration: 1	2	3	4	5	6
1	1.00	1.41	2.00	2.82	4.00	5.64
2	0.707	1.00	1.41	2.00	2.82	4.00
3	0.500	0.707	1.00	1.41	2.00	2.82
4	0.355	0.500	0.707	1.00	1.41	2.00
5	0.250	0.355	0.500	0.707	1.00	1.41
6	0.177	0.250	0.355	0.500	0.707	1.00

*If measurements are made at a gain setting different from that used to calibrate the threshold dial, the observed threshold reading must be corrected. To determine the appropriate factor, see gain setting being used and under the appropriate value of the calibration gain read the factor.

EXPERIMENTS

Calibration of the Threshold in Volume Units

The size distribution of erythrocytes of various species was determined as described (text-fig. 1) and these 50 percentile median points compared with the corresponding mean cell volume as determined by a hematocrit (text-fig. 2). The slope of the line drawn through these points was used as the factor to convert threshold dial readings to volume. To confirm this factor and the accuracy of the calibration factors used, a suspension of Krebs-2 ascites cells was sized with the counter and the volume distribution obtained compared with that from calculations based on diameters measured with an ocular micrometer (text-fig. 4). In contrast to the good agreement found between the electronic and optical measurements, the mean tumor cell volume calculated from packed-cell volume data was almost 40 percent greater than either of these two values (*13*, *14*). Presumably some of this discrepancy may be ascribed to the failure of spherical cells to pack tightly.

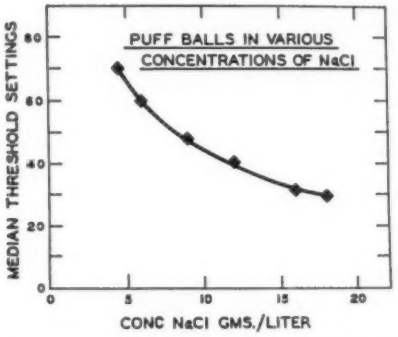


TEXT-FIGURE 4.—A comparison of volume distributions obtained from the electronic counter and volume distribution calculated from diameters measured in an ocular micrometer.

The mean cell volume of erythrocytes was estimated by integrating data from the counter and found to be about 3.7 percent greater than the median value. The corresponding correction was made in the factor even though this correction is certainly trivial for the present purposes.

Estimation of the Conductivity Factor

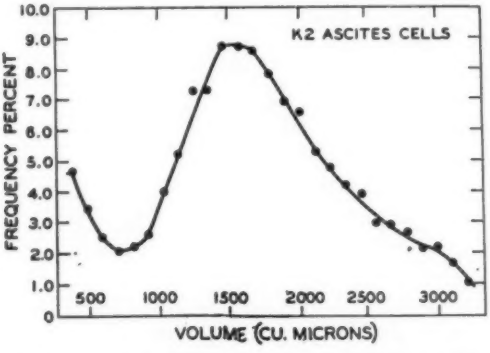
As indicated, changes in the conductivity of the counting medium gave rise to changes in the pulse size. Factors to correct for this effect were found by determining the median volume of puff balls in mediums of various tonicity. The puff balls themselves do not change in size under these circumstances, as shown by direct observation with ocular micrometer. The ratio (apparent volume/actual volume) may be used as a correction whenever the medium has a conductivity different from 0.9 percent NaCl (text-fig. 5).



TEXT-FIGURE 5.—The influence of the conductivity of the counting medium on the apparent median volume of a puff-ball suspension. Direct observation showed that the puff balls were not altered by exposure to these NaCl concentrations.

Tumor Cell Populations

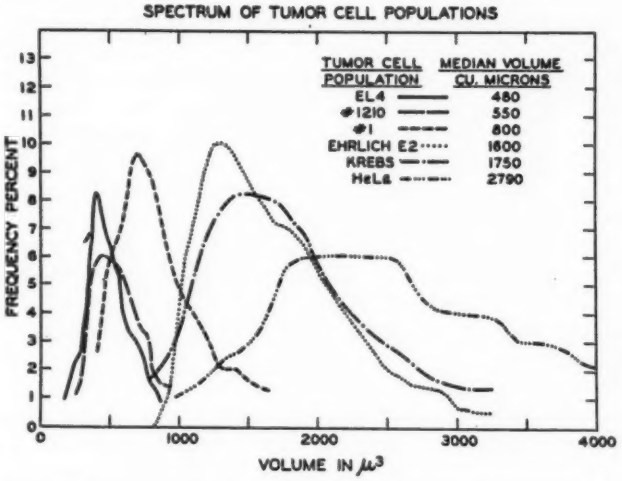
Although it had been anticipated that the cell sizes in a population of ascites tumors might change as the population aged (days after inoculation), there was no significant change in this regard. At 3, 5, 7, and 12 days after inoculation the average volumes (5 mice per group) were 1807, 1772, 1660, and 1707 μ^3, respectively. Text-figure 6 shows typical results.



TEXT-FIGURE 6.—Frequency distribution of volume in ascites carcinoma 5 days after transplantation.

The average of 20 determinations of the median volume was 1736.6 ± 73.4 μ^3 with a coefficient of variation of only 4.2 percent. This volume corresponds to a diameter of 14.88 μ.

Several other tumor cell populations were also studied by this method (text-fig. 7). In general these values are in agreement with values reported (*1, 15*).



TEXT-FIGURE 7.—Frequency distribution of volumes of several common ascites tumors, as well as the HeLa cell tissue culture.

Effect of Osmotic Strength of Medium on Size of Krebs-2 Ascites Cells

Krebs-2 ascites cells were studied with the counter in hypotonic and hypertonic media. The median values obtained were corrected for the changes in conductivity of the medium as described. Table 3 shows the marked changes in cell volume that occurred.

TABLE 3.—Volume of Krebs-2 ascites cells in hypertonic and hypotonic media

Concentration of NaCl in buffer (*percent*)	Volume μ^3	Diameter μ
0. 45	3480	18. 8
0. 90	1860	15. 2
1. 80	1130	12. 8

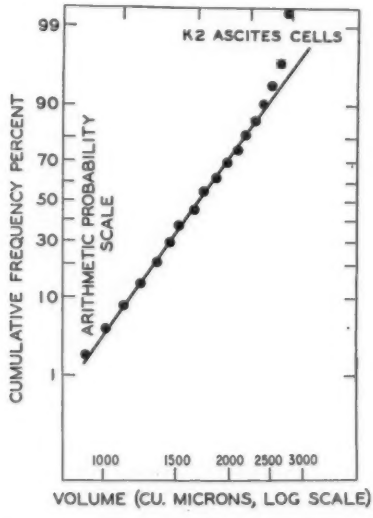
DISCUSSION

Although the Coulter counter gives information about size distribution, there is a question of whether the parameter involved is diameter, surface

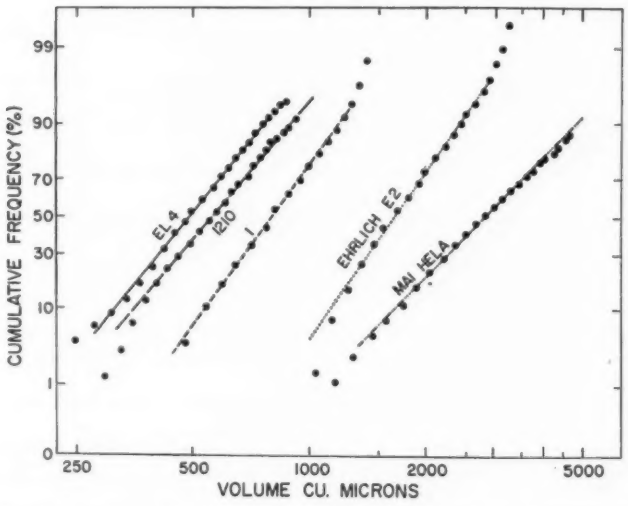
area, or volume. Kubitschek (*11*) has presented data which suggest quite clearly that the dimension measured is volume. The excellent correlation between volume and threshold (text-fig. 2) and the confirmation of the entire calibration based on volume (text-fig. 4) indicate that volume is in fact the parameter measured.

The volumes found for Krebs-2 ascites tumor cells do not change with tumor growth and are not normally distributed (text-fig. 6). Calculations reveal that neither volume, area, nor diameter are normally distributed. The logarithms of these parameters, however, are normally distributed. In text-figure 8 the cumulative frequency, plotted on a probability scale, versus the logarithm of the volume is a straight line, as required for a normal distribution. We are not able to account for this type of distribution, which has been found by others for many different cells (*16*, *17*). Nonetheless a plot of the data in this way reduces the many points to 2 parameters: the median log of volume, which is the mean of the logarithmic distribution, and the standard deviation, which is the reciprocal of the slope of the line. All the tumor cells studied possessed this distribution pattern and the standard deviation in each instance was about 40 percent (text-fig. 9). The distribution of volumes of erythrocytes did not follow this pattern.

To find the median (50 percentile point) from the counter data, it is necessary only to divide the total count by 2, and to locate the threshold value at which this value is plotted. There is some uncertainty concerning the true total count (100% point), inasmuch as there is a gradual slope instead of a flat plateau in this region which may be due to the



TEXT-FIGURE 8.—Same data as text-figure 6, plotted as cumulative frequency on arithmetic probability scale to show the normal distribution of the logarithm of the volume and indicating the coefficient of variation of the population.



TEXT-FIGURE 9.—Data of text-figure 7, plotted as cumulative frequency on arithmetic probability scale versus logarithm of volume.

presence of smaller cells and other unidentified particles (text-fig. 1). The 100 percentile point may be closely approximated by selecting the count at the threshold setting for which the derivative is a minimum (text-figs. 1 and 6). Because of the steepness of the curve in the region of the 50 percentile point, minor inaccuracies in the total count introduce little error in the estimation of the median volume.

Because of the cubic relation between diameter and volume, the corresponding diameter is obtained with even less error.

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Serial Passage of a Lymphocytic Tumor and Choriomeningitis Virus in Immune Mice [1]

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SUMMARY

Lymphocytic choriomeningitis (LCM) virus associated with an ascitic form of the lymphocytic tumor P288 survived indefinite serial passage in LCM-immune strain CDBA mice. Initially it was necessary to treat the mice with amethopterin (methotrexate), but after repeated passage the virus survived in untreated immune mice with intraperitoneal, but not with subcutaneous, tumors. In immune Swiss mice, where the tumor normally regresses, amethopterin enabled both tumor and virus to survive if the tumor was in the ascitic form. In immune Swiss mice not treated with amethopterin, the LCM-carrying tumor from 2 passage lines behaved differently; the tumor that had been maintained in amethopterin-treated immune CDBA mice did not support LCM survival, but the tumor adapted to passage in untreated immune CDBA mice supported LCM survival during the early stages of ascites tumor growth. The explanation offered for the survival of LCM in immune mice under these conditions is that virus passes from one tumor cell to its offspring at the time of cell division. Similarities between these LCM tumor-cell relationships and several other virus-cell interactions are discussed.—J. Nat. Cancer Inst. 25: 75–83, 1960.

THE VIRUS of lymphocytic choriomeningitis (LCM) can accompany certain transmissible murine tumors during serial transfers. Such virus-tumor associations have been found where the source of the virus was unknown (*1*); they have also followed intentional contamination of tumor lines, including amethopterin-(methotrexate) resistant tumors maintained in treated mice, with LCM (*2*). Ordinarily, passage of an LCM-carrying tumor through mice previously immunized against LCM eliminates the virus, but if the immune mice are treated with amethopterin, LCM may persist (*2*). This antimetabolite impedes development of immunity to LCM virus in mice, though it does not overcome an already established immunity (*3*). It also suppresses refractoriness of

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mice to several amethopterin-resistant transmissible tumors derived from foreign-strain hosts (*2*).

Experiments reported here show that certain combinations of amethopterin, LCM, and tumor enable the virus to survive prolonged serial passage in mice with fully developed immunity.

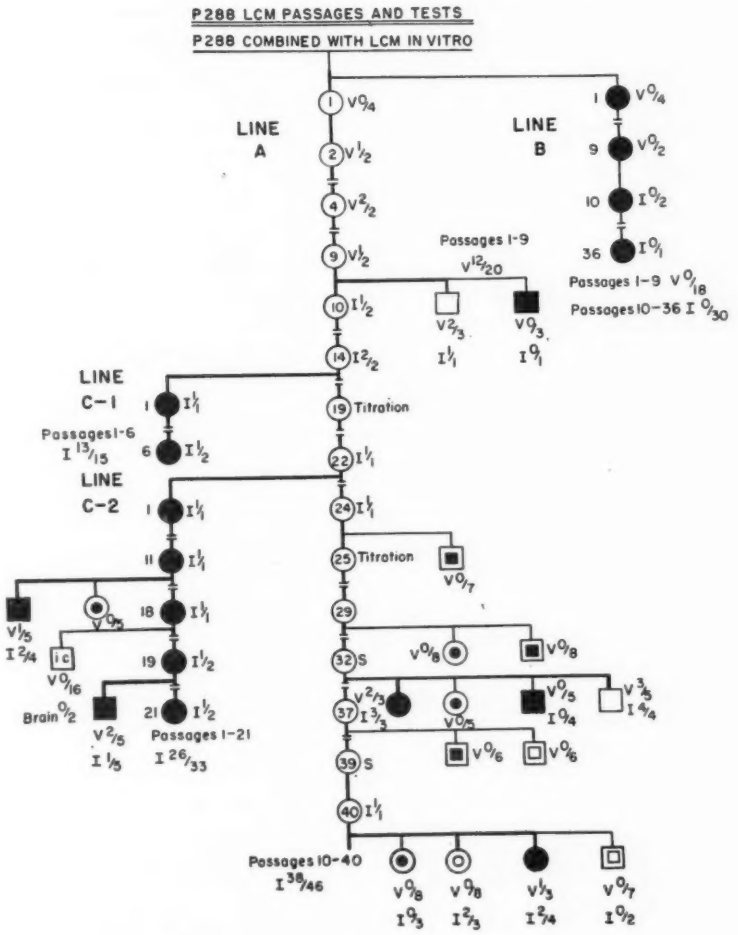
MATERIALS AND METHODS

Mice, either strain CFW or $CDBAF_1$ hybrids (of BALB/c × DBA/2), were obtained from the National Institutes of Health animal breeding facilities. The LCM (*4*) and the amethopterin-resistant lymphocytic tumor P288 (*5*) have been described. Mice were immunized by subcutaneous injection of live LCM (approximately 400 intracerebral LD50). This produced an inapparent infection, and mice survived intracerebral challenge 10 to 14 days later. Previous experience has shown that mice so inoculated with the strain of virus used do not become carriers (*3*). Virus-carrying tumor was established by mixing infected brain emulsion with ascites tumor *in vitro* and inoculating CDBA mice intraperitoneally with the mixture (*2*). Amethopterin was given subcutaneously on alternate days, in doses of 3 mg. per kg. of body weight. Testing for viremia was done by inoculation of heart blood intracerebrally into CFW mice (*3*). Additional details pertinent to particular phases of the studies appear under "Results."

RESULTS

The discussion of passages of virus-carrying tumor and tests for virus, presented in this section, may be followed from the diagram in text-figure 1A, which outlines most of the essential data.

Passage of LCM-carrying tumor.—An amethopterin-resistant line of tumor P288, in ascitic form, carrying LCM was transplanted for 40 generations in LCM-immune $CDBAF_1$ mice treated with amethopterin (line A, text-fig. 1A). Each mouse received 0.1 ml. of ascites diluted with an equal volume of saline (approximately 4–5 million cells) intraperitoneally; transfers were made at 5- or 6-day intervals. With rare exceptions, 2 donor and 2 recipient mice were used for each passage. During the first 9 passages viremia was found in 12 of 20 immune mice tested at the time of tumor transplant. Subsequently, virus was detected by injecting ascites (0.1 ml. diluted with an equal volume of saline) subcutaneously into CFW mice and challenging them intracerebrally 10 days later with 100 LD50 of stock LCM. The ascites produced a regressive tumor at the inoculation site; if LCM was present it immunized the mouse by inapparent infection. This test revealed virus in 38 of 46 ascites samples from the immune passage mice. In addition, ascites from the 32d and

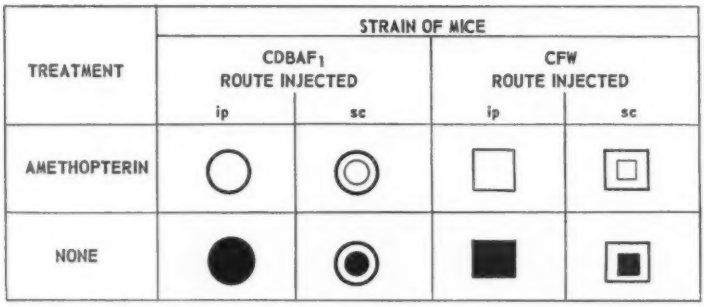


TEXT-FIGURE 1A.—Schematic presentation of passages of virus-carrying tumor and tests for virus.

39th passages, subjected to sonic disruption (15 min. in a 10 kc. Raytheon oscillator at 6° C.) to destroy the tumor cells, produced typical LCM in CFW mice injected intracerebrally.

Quantity of virus associated with tumor.—Ascites from the 19th passage immunized all CFW mice given dilutions of 10^{-1} and 10^{-2} and 2 out of 4 mice given 10^{-3}. At 10^{-4} it immunized only 1 out of 4.

Washed ascites cells from the 25th passage were approximately 1 log less potent. Resuspended to original volume, the washed cells numbered 37.2×10^6 per ml., which means that about every 3×10^4 cells carried a mouse-immunizing dose of LCM.



TEXT-FIGURE 1B.—Explanation of symbols used in text-figure 1A.

Unpublished experience in this laboratory and results reported by Rowe (*6*) indicate that the minimum infective dose of LCM for mice is of the same order of magnitude regardless of route of inoculation, but virus given intracerebrally produces fatal illness, whereas that introduced parenterally causes inapparent immunizing infection. Probably only a few virus particles are necessary to kill a mouse by the intracerebral route (LD50 of infected mouse-brain emulsion is about 10^{-6}); therefore, the mouse-immunizing dose should consist of relatively few particles too. This suggests that only a small proportion of ascites cells was associated with infectious virus, since 3×10^4 cells were required to provide an immunizing dose.

There is other evidence for this. Data in this report show a number of negative tests for virus, indicating that some mice in the virus-tumor passage series failed to develop detectable LCM infection. In a separate experiment, not reported in detail here, LCM was lost after 9 successful serial transfers of virus-carrying tumor in amethopterin-treated immune mice.

Initial necessity for amethopterin.—The virus-carrying tumor was injected into untreated immune $CDBAF_1$ mice at the time it was established in amethopterin-treated animals (line B, text-fig. 1A). During 36 passages in the untreated immune mice this tumor yielded no evidence of virus in 48 tests.

Later LCM survival without amethopterin.—After prolonged tumor-associated passage in amethopterin-treated immune mice, LCM was found to survive in untreated immune animals inoculated with the virus-carrying tumor. At the 14th passage in treated mice, ascites

passed intraperitoneally to untreated immune mice and carried for 6 generations yielded virus in 13 out of 15 samples tested during the 6 passages (line C-1, text-fig. 1A). Again, after 22 passages in amethopterin-treated immune mice, ascites was carried through untreated immune animals, this time for 21 generations, yielding virus in 26 out of 33 tests (line C-2, text-fig. 1A).

For LCM to survive in untreated immune $CDBAF_1$ mice the tumor had to be in ascitic form. Subcutaneous injection of ascites from the 29th and the 32d passages of the amethopterin-treated series (line A, text-fig. 1A) into untreated immune mice produced tumors at the inoculation site but no detectable viremia in 13 tests during the 5th to the 9th days. For comparison, some of the ascites from the 32d passage, given intraperitoneally to untreated $CDBAF_1$ immunes, produced both viremia and virus-carrying ascites. The second LCM-carrying tumor line established in untreated immune mice, referred to in the preceding paragraph (line C-2, text-fig. 1A), also failed to support virus survival when given subcutaneously. After 11 serial passages by the intraperitoneal route this tumor was inoculated into untreated immunes subcutaneously; the mice developed tumors but no viremia was demonstrable in 5 tests.

Another attempt to recover LCM from untreated immune mice with subcutaneous tumors was made after the virus-carrying ascitic tumor had been passed 40 times through amethopterin-treated mice (line A, text-fig. 1A). Infected ascites was given to one group of untreated immunes subcutaneously and to another intraperitoneally. The mice inoculated subcutaneously yielded no virus in 8 tests on blood and 3 on the minced tumors, but those inoculated intraperitoneally produced viremia in 1 of 3 tests, and virus was detected in their ascitic tumors in 2 of 4 samples. A third group of immune mice, inoculated subcutaneously but treated with amethopterin, yielded LCM twice in 3 tests of solid tumors.

It is evident that initially the survival of LCM in immune mice inoculated with virus-carrying ascitic tumor required that the mice be treated with amethopterin (*cf.* line A, text-fig. 1A, with line B); later, after a number of such passages, amethopterin was no longer necessary (lines C-1 and C-2). But the virus did not survive in immune mice bearing the tumor in subcutaneous form unless amethopterin was given (tests from line A, shown in the text-fig. at passages 29, 32, and 40, and line C-2 at passage 11).

The LCM which accompanied ascites tumor through 22 passages in amethopterin-treated immune mice, then through 18 passages in untreated immunes (line C-2, text-fig. 1A), was inoculated intracerebrally into immune and nonimmune CFW mice after treatment in the sonic oscillator to disrupt the tumor cells. In nonimmune mice this virus produced typical, fatal LCM; in immunes it produced no illness or death, and no virus was demonstrable in blood or brains, even in a group treated with amethopterin. (Data shown in text-fig. 1A are for the untreated immunes.) This is taken to mean that survival of this strain

of LCM in untreated immune tumor-bearing mice was not due to an acquired ability of the virus itself to withstand the hosts' immune reaction.

The ability to support LCM survival in ascites-bearing untreated immune mice did not appear in lines of virus-carrying P288 maintained by serial passage in nonimmune mice (not shown in text-fig. 1A). In one instance, after such a line of ascites was found to have been contaminated with LCM unintentionally, 3 passages through untreated immune mice eliminated demonstrable virus from the tumor. In another instance, LCM which had accompanied 2 ascites lines through 38 transfer generations in nonimmune mice was eliminated from one line by a single passage through immune mice and from the other by 2 such passages.

LCM in tumor-bearing immune CFW mice.—Since P288 supports LCM survival in immune $CDBAF_1$ mice, it ought to do likewise in another strain if the tumor can be made to survive in that strain. Ordinarily P288 regresses in CFW mice, and 2 attempts to establish LCM-carrying ascites in untreated LCM-immune CFW mice yielded neither viremia nor infected ascites in 13 tests during the 5th to the 9th days. These attempts were made with LCM-carrying tumor after 9 and 32 serial passages through amethopterin-treated immune $CDBAF_1$ mice (line A, text-fig. 1A).

Treatment of CFW mice with amethopterin promoted survival of P288 in ascitic form throughout indefinite serial passage. In a preliminary experiment, it was maintained through 26 transfers in treated CFW mice, killing 92 out of 104 inoculated. Ascites from mice of this series, taken at the 8th, 9th, and 21st passages, produced typical, fatal ascitic tumors in $CDBAF_1$ mice. In untreated CFW mice the tumor survived through 5 generations when passed at 6- to 9-day intervals, but 21 subsequent passages of peritoneal washings yielded no material capable of killing either CFW or $CDBAF_1$ mice. Even the tumor maintained in amethopterin-treated CFW mice had not, after 22 passages in this strain, acquired the ability to survive in untreated animals.

Virus-carrying P288 from the amethopterin-treated immune $CDBAF_1$ passage series (line A, text-fig. 1A) was twice inoculated intraperitoneally into amethopterin-treated LCM-immune CFW mice, once after 9 passages in the $CDBAF_1$ series and again after 32 passages. In both instances tests on blood and ascites yielded virus in 10 out of a total of 13 samples. Here the amethopterin enabled the tumor to survive in a refractory host and simultaneously allowed its associated LCM to persist in spite of the mouse's immunity. But tumor from this passage line (line A) inoculated subcutaneously into immune CFW mice at the 24th, 29th, 37th, and 40th transfer generations produced no viremia in 34 tests, of which 13 were from mice treated with amethopterin; 2 tests of tumors at the site of inoculation in the amethopterin-treated CFW mice yielded no virus.

The line of LCM-carrying ascites adapted to passage in untreated immune $CDBAF_1$ mice (line C-2, text-fig. 1A) was inoculated intraperitoneally into untreated immune CFW mice after 11 passages in the un-

treated immune $CDBAF_1$ series, and again after 19 such passages. These untreated immune CFW mice yielded viremia and virus-infected ascites in 6 of 10 tests on the 5th and 6th days, this being the period before tumor regression began; 9 tests on the 7th and 8th days were all negative. In thus supporting LCM survival in untreated LCM-immune CFW mice, this line of virus-carrying P288, which had been passed many times in untreated immune $CDBAF_1$ mice, differed from the line maintained in treated animals (line A, text-fig. 1A).

DISCUSSION

Under the conditions of these experiments the ascitic form of the lymphocytic tumor P288 tended to shelter LCM from the antiviral mechanism of immunized $CDBAF_1$ mice during prolonged serial passage by intraperitoneal inoculation. Initially this required that the mice be treated with amethopterin, an antagonist of nucleic acid synthesis, which is known to interfere with the mouse's immune response to LCM, but during passage in treated immune mice, the virus-tumor association changed in the direction of enabling LCM to survive through many serial passages in untreated immunes.

In immune $CDBAF_1$ mice inoculated subcutaneously with LCM-carrying P288, virus was recovered only if the mice were treated with amethopterin.

Amethopterin enabled both the ascitic tumor and its associated LCM to survive in immunized CFW mice but no evidence of LCM survival was found in immune CFW mice with tumors at the site of subcutaneous inoculation. The line of virus-carrying ascites adapted to passage in untreated immune $CDBAF_1$ mice supported LCM survival in untreated immune CFW mice during the first 6 days of intraperitoneal tumor growth before regression developed.

The immune mice used in these experiments had withstood intracerebral challenge with homologous virus before they were inoculated with LCM-carrying tumor. Yet despite their firmly established immunity, they did not eliminate LCM from certain virus-tumor associations. Initially, amethopterin doubtless interfered with the immune mechanism just enough to allow LCM to survive under the sheltering influence of the tumor, but later this sheltering influence became effective enough to foster virus survival in immune mice without amethopterin.

The LCM survival described here could have depended upon passage of virus from infected tumor cells to their offspring during cell division. Under such conditions the virus inside the tumor cells might be isolated from the mouse's immune mechanism. That only a small proportion of tumor cells appeared to carry infective virus with them favors such a concept. The occurrence of virus in blood and the recovery of less virus from washed cells than from whole ascites suggest that some virus release from tumor cells took place, but virus survival in a rapidly dividing tumor need not have depended upon the entrance of this released virus into new cells if virus passed from mother to daughter cells.

The fact that the virus-tumor association became more effective in supporting virus survival during passage in treated immune mice could mean that synchronization of virus replication and tumor-cell division developed as virus-tumor association was prolonged. If virus survival depended on its transfer during cell division, then it would be necessary for virus replication to be at such a stage at the time of cell division that potent virus material would be available to pass on to the descendants of the infected cell. Lack of synchronization would probably lead to premature virus release and consequent exposure to the mouse's immune mechanism. It can be assumed that a certain amount of this did occur but that enough synchronization developed to allow virus survival.

While this hypothesis could account for the behavior of the virus-carrying ascitic tumor, it does not explain why virus failed to survive in untreated immune mice when the tumor was inoculated subcutaneously instead of intraperitoneally, nor why the subcutaneous tumor failed to support LCM survival in immune CFW mice even when they were treated with amethopterin. One possible explanation might be that the mouse's mechanism of immunity against this virus was not as effective within the peritoneal cavity as it was in the subcutaneous tissues. Another could be that metabolic conditions prevailing in subcutaneous tumors are sufficiently different from those in the ascitic forms that they influence the ability of the tumor to support virus replication in an immune host. In any event, the weakening of the mouse's immune response by amethopterin appeared to contribute significantly to virus survival under the conditions of these experiments.

Although the experimentally determined facts do not prove that LCM survival in these immune tumor-bearing mice depended upon passage of the virus during cell division, they at least show that virus and tumor became closely associated and that this association was strengthened, by suitable serial passage, to the point where amethopterin was no longer necessary for virus survival in immune mice bearing the ascitic tumor. It would be easy to assume that the tumor supported virus survival by providing an endless supply of susceptible cells rather than by carrying virus along at the time of cell division, but that hypothesis would not explain the strengthening of the virus-tumor association which developed during their passage together.

The LCM-tumor relationship observed here recalls the description by Rubin and Temin (*7*) of initiation of foci of Rous sarcoma cells. Such foci were made by both cell multiplication and virus release, individual tumor cells having the ability to carry on dual activities of cell multiplication and virus production. Only a fraction of the cells were competent at any one time to support virus replication. The major role in development of foci was thought to depend on cell division.

Certain resemblances to carrier states in tissue cultures of non-tumor viruses merit attention here, also. Lwoff originally proposed the term to identify mixed populations of bacteria and bacteriophage "in a more or less stable equilibrium" (*8*), and Ackermann applied it to HeLa cell

cultures of poliovirus, where the virus-cell relationship depended on the presence of immune serum in the medium (*9*). In the studies reported here, the virus-tumor relationship developed in mice fully immunized against LCM.

In a carrier culture of Coxsackie A9 virus in HeLa cells, Takemoto and Habel (*10*) observed no evidence of lysogeny and found only a small proportion of cells infected, the latter being a point of similarity to the LCM-tumor cell association described here. They noted that rapid thermal inactivation prevented extensive virus multiplication; in the present studies host immunity appears to have produced a similar effect.

These resemblances of the LCM-tumor cell relationship to the carrier states appear even more clear if one regards the conditions described in this report as constituting an *in vivo* tissue culture. The immune mouse provides an environment that permits survival of an abundance of tissue-culture (*i.e.*, tumor) cells but one that is specifically unfavorable for the virus.

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Chromosome Studies on Normal and Leukemic Human Leukocytes [1]

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SUMMARY

Chromosome studies were made on myeloblasts obtained from the peripheral blood of 4 patients (3 males, 1 female) with acute or chronic granulocytic leukemia and on leukocytes from 3 healthy individuals (1 male, 2 females). The cells were grown in culture for 2 to 4 days before examination. Both in normal and in leukemic cells, the predominant metaphase chromosome number was 46. However, idiogram analyses revealed a definite abnormality, probably involving the Y chromosome, in the 2 cases of chronic granulocytic leukemia. No chromosome abnormality was distinguishable in the 2 cases of acute leukemia (1 male, 1 female) investigated. The results indicate that in some leukemic human leukocytes small but definite chromosomal changes are demonstrable.—J. Nat. Cancer Inst. 25: 85–109, 1960.

ALTHOUGH THERE have been numerous reports in recent years of chromosome abnormalities in a wide variety of malignant tumors, there is very little information on the chromosomes of leukemic cells in man. A few workers (*1*, *2*) have observed mitotic figures in direct smears or in tissue cultures of cells from the blood or bone marrow of patients with leukemia and have noted that in general the chromosome number appeared to be in the diploid range; earlier, Andres and Shiwago (*3*) described mitotic variations in leukemic cells similar to those seen in other malignant cells. However, until three very recent reports, no exact counts or analyses had been made.

Nowell *et al.* (*4*) obtained myeloblasts from the peripheral blood of 3 patients with acute granulocytic leukemia and, after short-term culture, found that the chromosome number agreed with the diploid value for man ($2n = 46$) and that no gross chromosomal abnormalities were present. More recently, Ford *et al.* (*5*) reported similar results from studies of bone marrow in short-term culture in one case of lymphocytic leukemia and

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in another of blast-cell leukemia, but, in a third leukemia, of unspecified type, an abnormal number of 44 chromosomes plus 1 minute was observed. Baikie *et al.* (*6*) have just described chromosome studies in 11 cases of leukemia, employing the bone marrow technique. No aberrations were found in preliminary investigation of 6 cases of chronic leukemia, but in 5 cases of acute leukemia, there was abnormality of chromosome number in 1 case, abnormality of chromosome morphology in 2 cases, and abnormality of both number and morphology in the 1 case reported in detail.

In the present study, which is an extension of our previous report, the chromosomal characteristics of both normal and leukemic human leukocytes have been investigated by the use of short-term primary cell cultures, and it is believed, for reasons which will be discussed, that the results obtained accurately reflect the state *in vivo*. One of the 3 normal individuals described here (Normal case 1) was included in our earlier abstract, as were the 2 acute leukemia cases (1 and 2). In these cases, the preliminary data have been confirmed and amplified. Two additional normal cases and and 2 chronic leukemia cases are described here for the first time.

MATERIALS AND METHODS

Leukocytes were separated with phytohemagglutinin (Difco) from heparinized peripheral blood of healthy individuals and of patients with granulocytic leukemia. The cells were grown in short-term culture by a modification of Osgood's "gradient" method, which has been reported in detail elsewhere (*7*, *8*). Briefly, cells were grown in undisturbed deep cultures in a mixture of autogenous plasma and commercial medium (TC-199, Difco). Each culture bottle contained 1 or 2 slanted slides, and the cells settled out and grew on the slides as well as on the sides and bottom of the bottle. Mitoses appeared in the leukemia cultures on the 1st or 2d day, but were rarely observed in the cultures from normal individuals before the 3d day. In the leukemia cultures, nearly all of the inoculated cells were immature myeloid forms and were the source of the mitotic figures studied. The dividing cells in the normal cultures were derived from monocytes and perhaps large lymphocytes (*7*, *8*). Recent work (*9*) indicates that the phytohemagglutinin used in separating the leukocytes from whole blood is a specific initiator of mitotic activity among the normal leukocytes in these cultures.

Colchicine (1×10^{-7}M) was added at the time the cells were undergoing their first division in culture. Seventeen to 19 hours later, the cells were harvested from the bottom of the culture bottle, pretreated, fixed and stained in acetic orcein, and squash preparations were made (*7*, *10*). At the same time, the slides from the culture bottles were rinsed in distilled water, air-dried, and stained with Giemsa. The slides were used only for general survey purposes, while the actual chromosome counts and analyses were made on the squash preparations. In some instances, colchicine was

not used, so that anaphases and details of uncontracted metaphase chromosomes could be examined.

Idiograms were constructed from enlarged photomicrographs. In each case, the chromosomes have been classified into 3 groups, according to centromere position: median (top row, 7 pairs), subterminal (middle row, 6 pairs), and submedian (bottom row, 10 pairs). Within each of the 3 groups, the pairs have been arranged according to approximate order of decreasing length.

CASE HISTORIES

Chromosome studies were made on the leukocytes of 4 patients with acute or chronic granulocytic leukemia and 3 healthy individuals, members of the laboratory staff. Brief histories of the leukemia cases follow:

Leukemia case 1.—E. C., a 59-year-old white male, was admitted to Philadelphia General Hospital on July 25, 1957, because of weakness and fever of 1 month's duration. WBC = 37,000 (64% blasts); bone marrow diagnosis: "acute granulocytic leukemia." He was treated with 6-mercaptopurine and steroids during July and August, 1957, and from November, 1957, until his death, but never attained complete remission. White counts fluctuated from 3,000 to 130,000, with a high percentage of blast forms at all times. He died on April 17, 1958, after recurrent episodes of hemorrhage and infection. A total of 3 blood samples was obtained for chromosome study from this patient: 2 midway in the course of his disease (November 22, 1957, and December 2, 1957) and 1 during the terminal stages (March 28, 1958). White blood cell counts at those times were, respectively, 30,000 (92% blasts); 35,000 (88% blasts); and 52,000 (84% blasts).

Leukemia case 2.—I. K., a 35-year-old white female, was admitted to the Hospital of the University of Pennsylvania on October 12, 1957, with a history of weakness and purpura for 3 weeks. WBC = 12,000 (85% blasts and promyelocytes); bone marrow diagnosis: "acute granulocytic leukemia." Treatment with 6-mercaptopurine and steroids was started but was without effect. She died on October 16, 1957, with multiple hemorrhages. A blood sample for chromosome study was obtained on October 14, 1957; at that time WBC = 11,000 (91% blasts and promyelocytes).

Leukemia case 3.—E. K., a 41-year-old white male, whose case was first diagnosed as "chronic granulocytic leukemia" in April, 1955 (WBC = 318,000), was treated with Myleran and a remission was obtained which lasted until February, 1957. Exacerbation of the leukemia at that time was treated, with only partial success, by X-ray therapy to the long bones and with P^{32}. He remained reasonably well until July, 1958, when a second exacerbation of the disease failed to respond to further X-ray and Myleran therapy. He was admitted to the Hospital of the University of Pennsylvania August 13, 1958, and bone marrow examination confirmed the diagnosis of chronic granulocytic leukemia. He died of cerebral hemorrhage on August 27, 1958. A blood sample for chromosome study was obtained on August 19, 1958; at that time WBC = 375,000 (blasts and promyelocytes, 16%; myelocytes, 37%; metamyelocytes, 33%; segmenters, 12%; lymphocytes, 2%; monocytes, 0%).

Leukemia case 4.—G. H., a 33-year-old Negro male, was admitted to Philadelphia General Hospital on August 13, 1958, with a history of weakness and fatigue for 2 months. WBC = 248,000; bone marrow diagnosis: "chronic granulocytic leukemia." Myleran therapy was started on August 22, 1958. He was discharged, in remission, on September 17, 1958, and lost to follow-up. Blood for chromosome study was obtained on August 25, 1958; at that time WBC = 222,000 (blasts and promyelocytes, 17%; myelocytes, 16%; metamyelocytes, 14%; bands and segmenters, 31%; eosinophils, 7%; basophils, 10%; lymphocytes, 5%; monocytes, 0%).

RESULTS

Preliminary study of Giemsa-stained slides from both leukemia and normal cultures, neither of which had received colchicine, failed to reveal any gross mitotic abnormalities. Anaphases were entirely regular, without lagging or bridging of chromosomes. There were no spindle abnormalities such as multipolarity. Metaphase chromosome numbers were predominantly in the diploid range. Approximate counts of 200 metaphases on one slide (Leukemia case 1) gave a mean value of 44 chromosomes, with a standard error of $\pm$ 4. In both the normal and the leukemia cultures, approximately 2 percent of the metaphases were in the tetraploid range.

More precise studies on the squash preparations confirmed the impressions gained from examination of the Giemsa-stained culture slides. Chromosome counts were done on at least 30 metaphases in each case (tables 1 and 2); only those counts of metaphases which were accurate within 1 chromosome were accepted. A count recorded in the tables as 45 ± 1, 46 ± 1, 47 ± 1, or 48 ± 1 indicates that at one place in the metaphase plate it could not be definitely decided whether 1 or 2 chromosomes were present. The predominant chromosome number was 46 in all cases, both normal and leukemic.

TABLE 1.—Metaphase chromosome numbers of leukocytes from peripheral blood of normal humans

Case number	Chromosome number					
	45	45 ± 1	46	46 ± 1	47 ± 1	47
1	3	3	25	4	4	—
2	—	3	32	2	1	3
3	—	2	26	1	3	—

TABLE 2.—Metaphase chromosome numbers of leukocytes from peripheral blood of leukemic humans

Case number	Chromosome number						
	44	45 ± 1	46	46 ± 1	47 ± 1	47	48 ± 1
1	—	11	21	5	5	3	—
2	—	10	13	5	6	—	—
3	1	9	11	2	7	—	—
4	—	8	16	7	9	1	1

The tetraploidy which had been observed in both the normal and leukemia cultures was found, in the squash preparations, to be of two types, one showing endoreduplication (*11*) and one not; none of these was accurately countable and they are not shown in the tables. In addition, occasional apparently intact metaphases were observed in colchicine-treated material, *both* normal and leukemic, which had markedly *hypo-*

diploid chromosome numbers. Such a cell, with 28 chromosomes, is shown in figure 1. In one individual, not included in this report, idiogram analyses were made of 3 such metaphases, each having 10 chromosomes. There was little or no correspondence of chromosome types among the 3 and, in general, no pattern either of chromosome number or type was observed in other hypodiploid metaphases; their chromosome complements appeared to be derived at random from the normal complement. Since these hypodiploid forms were never observed in the absence of colchicine, it is thought that they represent a phenomenon induced by the relatively long colchicine treatments employed. Study of culture slides on which such cells appeared suggested that they resulted from a type of segmentation of cells arrested at metaphase. A stage in this process is shown in figure 2, a photomicrograph from a squash preparation; the 2 groups of chromosomes total 46.

These types of variation in chromosome number occurred with equal frequency in both normal and leukemic cultures. Of greater interest are the results of idiogram analysis, which revealed abnormalities of chromosome morphology apparently peculiar to cells in the 2 cases of chronic leukemia. Detailed descriptions of the results in each case follow.

Normal case 1.—White female, age 21. Metaphase chromosome numbers predominantly 46. Five idiogram analyses were made of cells with 46 chromosomes. Four of these were normal (figs. 3, 7), and 1 showed an abnormally long chromosome, apparently the result of a translocation. Of a total of 39 metaphases counted, 3 had 45 chromosomes. One of these was analyzed, and it was found that the missing chromosome belonged to the fifth or sixth longest pair of the group with submedian centromeres.

Normal case 2.—White female, age 48. Metaphase chromosome numbers predominantly 46. Three idiogram analyses were made of such cells and all were normal (figs. 4, 8). Of a total of 41 metaphases counted, 3 had 47 chromosomes. One of these was analyzed and found to have a large acentric or telocentric fragment in addition to a normal female complement (fig. 5).

Normal case 3.—Japanese male, age 31. Metaphase chromosome numbers exclusively 46, within the adopted limits of accuracy. A total of 31 metaphases was counted, of which 3 were analyzed (figs. 6, 9). No morphological irregularities were observed, and the Y chromosome (fig. 20a–c) compares well with descriptions of this chromosome by other workers (*5, 13, 14*).

Leukemia case 1.—White male, age 60. Metaphase chromosome numbers predominantly 46. Of 45 metaphases counted, 3 had 47 chromosomes. One of these 3 was analyzed, and an apparently normal complement was present, plus 1 minute chromosome. Five idiogram analyses were made of cells with 46 chromosomes and all were indistinguishable from normal (figs. 10,15, 21a–c).

Leukemia case 2.—White female, age 35. Metaphase chromosome numbers exclusively 46, within the error tolerated. Thirty-four met-

taphases were counted and 4 idiogram analyses were made; all were indistinguishable from normal (figs. 11, 16).

Leukemia case 3.—White male, age 41. Thirty metaphases were counted. Chromosome numbers were 46, within error tolerated, with the exception of a single count of 44 (idiogram analysis not possible). Analyses were made of 3 cells with 46 chromosomes. All idiograms revealed the absence of a normal Y and the presence, in its stead, of a chromosome less than half the size of the smallest autosomes (figs. 12, 17, 22a–c). This minute chromosome was recognized in many other metaphases that were not subjected to idiogram analysis.

Leukemia case 4.—Negro male, age 33. In 42 metaphases counted, chromosome numbers were predominantly 46, with 1 count of 48 ± 1 (idiogram not possible). Analyses were made of 5 cells with 46 chromosomes. One idiogram (figs. 14, 19) showed an apparently normal Y chromosome. The other 4 idiograms, however, showed, instead of the Y, a minute chromosome (figs. 13, 18, 23a–c) similar to that observed in Leukemia case 3.

Figures 20 to 23 provide a comparison of Normal case 3 (male) with Leukemia cases 1, 3, and 4 (males). The comparison is made to illustrate the relative sizes of the two smallest pairs of acrocentric chromosomes and the Y chromosome or the minute chromosome, whichever was present in each metaphase.

DISCUSSION

Although the metaphase chromosome numbers were predominantly 46 in all 4 cases of granulocytic leukemia investigated, definite abnormalities of chromosome morphology were present in 2 of the individuals. In the 2 cases of chronic leukemia in males, idiogram analyses revealed changes of a similar type involving the presence of a minute chromosome. These minutes, which were morphologically similar but not identical in the 2 cases, were apparently present in place of a normal Y, and are judged to have resulted from either a deletion of a portion of the Y chromosome or the replacement of the Y by an autosomal fragment. The possibility that the minute simply represents normal variation in the morphology of the Y chromosome seems unlikely since studies by other workers have not revealed such variation (*12*, *13*). Nor does it seem likely that the minute resulted from an abnormality of 1 of the 4 smallest acrocentric autosomes rather than of the Y, since none of the chromosomes interpreted in our material as being these 4 autosomes resembled a normal Y, such as was observed in our Normal case 3 and has been described by others (*12*, *13*). Interestingly, if the change observed does involve the Y chromosome, it is not correlated with sex linkage in the incidence of chronic granulocytic leukemia, since statistical studies show only slightly greater frequency in males than in females (*14*).

The chromosomes of the 2 cases of acute leukemia (1 male, 1 female) were indistinguishable from normal with respect to both number and

constitution. However, in the female, an alteration in chromosome morphology of the order of magnitude of that observed in the Y chromosome of the chronic leukemias might well be impossible to detect. In this connection it is, of course, possible that a variety of subtle cytogenetic changes [the "cryptostructural" rearrangements of Levan (*15*)] as well as purely genetic changes might be present in any of these leukemic cases and be undetectable by our methods.

On the basis of the few cases studied, no estimate can be made regarding the possible relationship of chromosome changes to the duration of the disease process or to the duration or type of therapy. However, in Leukemia case 1 no chromosome changes were present even after prolonged chemotherapy, while in Leukemia case 4 the minute chromosome was present although treatment had just been started. In the one case reported in detail by Baikie *et al.* (*6*), a female with acute granulocytic leukemia, the modal chromosome number changed after 7 months of steroid therapy, but abnormalities of both number and morphology were present before treatment was begun.

There seems little doubt that the "leukemic" chromosome patterns studied in the present paper were indeed derived from leukemic cells. Nearly all of the cells planted in the cultures from leukemic patients were immature myeloid forms; monocytes and large lymphocytes, the cells which divide in *normal* cultures, constituted no more than 5 percent of the original inoculum. Furthermore, the leukemic mitoses studied had usually occurred during the first 2 days in culture, whereas normal leukocytes were rarely observed in mitosis before the 3d day of culture. Finally, when permitted to mature *in vitro*, the dividing cells in the leukemia cultures developed into polymorphonuclear forms, while the dividing cells in the normal cultures differentiated into macrophages, multinucleated giant cells, and small lymphocytes (*8*).

The present study also provides data on the chromosomes of leukocytes of three normal individuals, one male and two female. Of particular interest in these cases is the low but definite incidence of chromosome abnormalities in these normal cells. Such abnormalities among other populations of normal human somatic cells have been reported previously by other authors. Counts other than euploid are regarded by Ford *et al.* (*5*) as artifacts of technique or interpretation. Tjio and Puck (*12*) state that of 2,000 cells, 99.9 percent had chromosome numbers of 46 or 92, with a tetraploid frequency of less than 3 percent. Chu and Giles (*13*) mention that in a few exceptional cells deviations of one chromosome from normal were observed. These were interpreted as representing either instances of somatic aneuploidy, originally present or induced during culture, or artifacts resulting from errors in technique. In the three normal cases presented here, there is no suspicion of pathological origin of the cells studied. The chance that such aneuploid numbers were induced during culture is remote, since only the first or, at the very most, the second mitosis *in vitro* is represented in these preparations. We believe that technical and interpretive errors have been

ruled out in the cases of aneuploidy and other chromosome aberrations in which idiogram analyses were made. While, as mentioned, it is not possible to estimate reliably in our preparations the frequency of such aberrations, it is reasonable to believe it is greater than 0.1 percent and probably not more than 2 percent.

The relationship of the present findings in human leukemia to the results of such investigations on other types of malignant tumors is not yet clear. Most previous studies of spontaneous mammalian cancers have revealed much greater variability of chromosome number and morphology than that reported here [*see*, for example Hsu (*16*), Koller (*17*)]. Leukemic cells are apparently not so far removed from their normal prototypes as the cells of many types of malignant tumors. For instance, leukemic "blasts" are essentially indistinguishable, both morphologically and biochemically (*18*), from the "blasts" of normal bone marrow, and *normal* as well as leukemic leukocytes have the capability, present only in *malignant* cells of other tissues, of detaching from their fellows and spreading throughout the body. Whether the more subtle chromosome changes observed in leukemic cells, as compared to other types of malignancies, are related to these biological differences has not yet been determined. Furthermore, the relationship of our results to those of Baikie *et al.* (*6*), who observed chromosomal abnormalities in acute leukemia, but not in chronic leukemia, cannot as yet be evaluated. The studies of chronic leukemia reported by Baikie *et al.* (*6*) were of a preliminary nature and might not have revealed the small chromosome abnormality observed in our chronic cases. Obviously, more extensive studies are needed on the chromosomes of various types of human leukemia and lymphoma, and such studies are planned. In addition, cytological investigations of other early primary neoplasms, both benign and malignant, such as the recent study by Palmer (*19*) of the Shope rabbit papilloma, may lead to a better understanding of the relationship of chromosome changes to the pathogenesis of tumors.

Notes added in proof:

1) A recent paper by Bayreuther (Nature 186: 6–9, 1960) indicates that the incidence of chromosome aberrations in a variety of early spontaneous and induced malignancies may not be as great as indicated by other workers.

2) A more detailed analysis of chromosomes in human leukemia will be provided by studies now in progress in our laboratories, employing an improved method of chromosome preparation from leukocyte cultures (Moorhead, P. S., Nowell, P. C., Mellman, W. J., Batipps, D. M., and Hungerford, D. A.: To be published).

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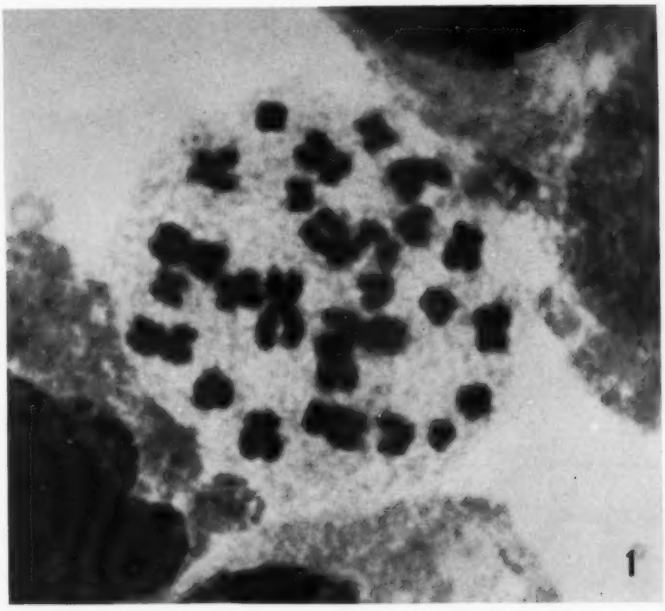
PLATE 11

FIGURE 1.—An apparently intact metaphase chromosome group with 28 chromosomes. Such metaphases were not observed in the absence of 17- to 19-hour colchicine treatment. × 2,700

FIGURE 2.—A stage in the formation of hypodiploid chromosome groups. The two groups of chromosomes total 46. × 2,700



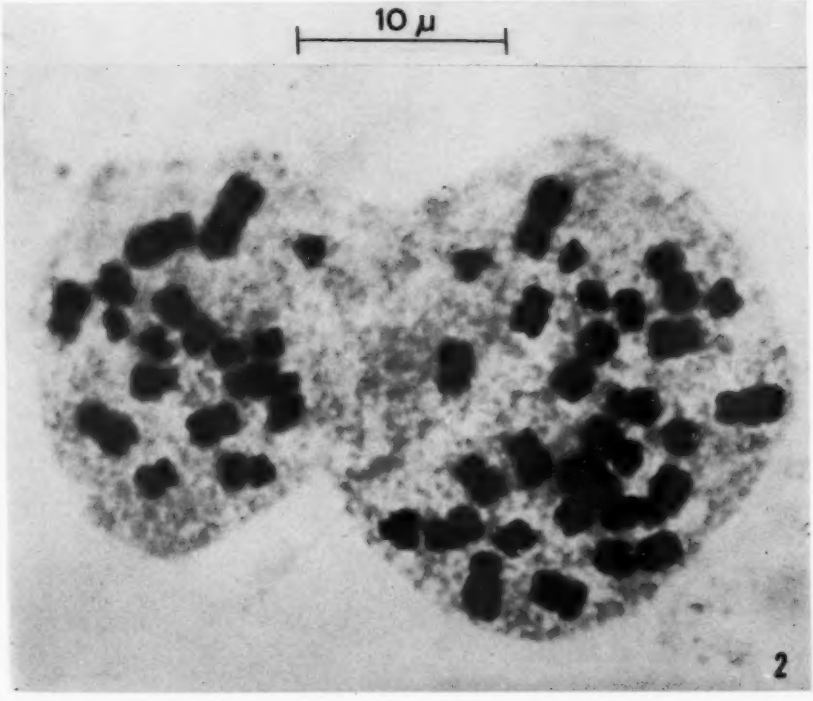


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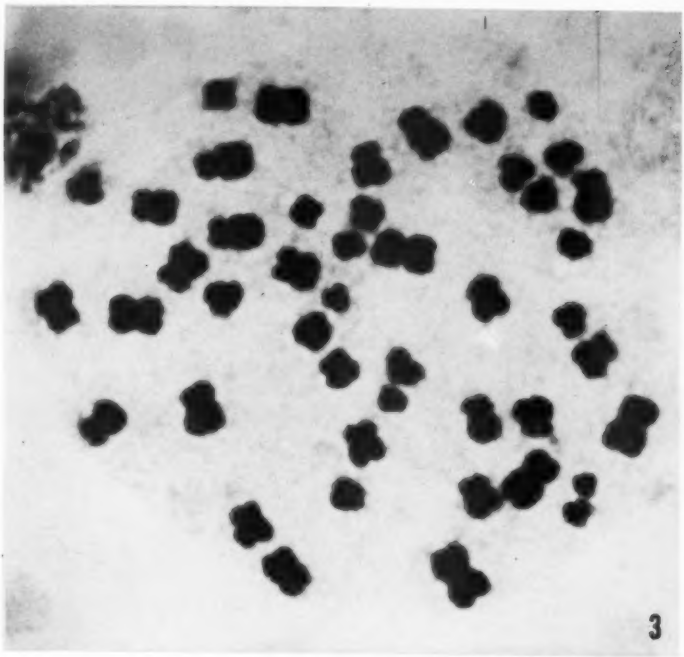
PLATE 12

FIGURE 3.—Metaphase from Normal case 1 (female), 46 chromosomes. *See* idiogram, figure 7. × 2,700

FIGURE 4.—Metaphase from Normal case 2 (female), 46 chromosomes. *See* idiogram, figure 8. × 2,700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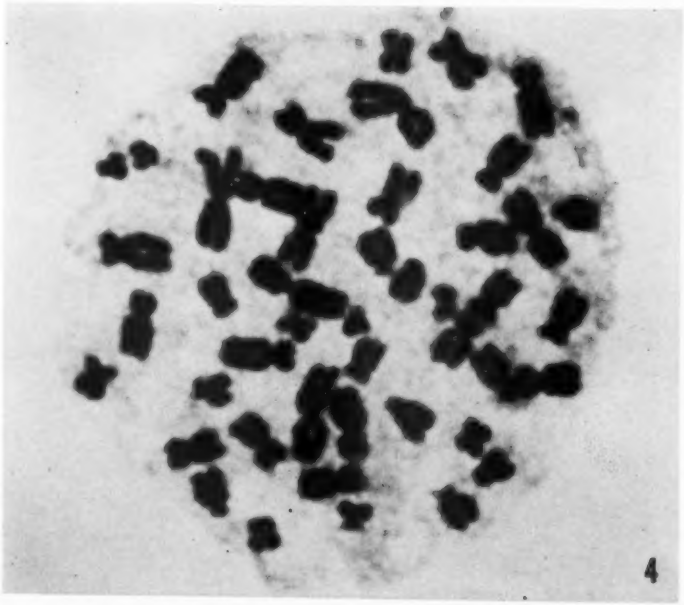
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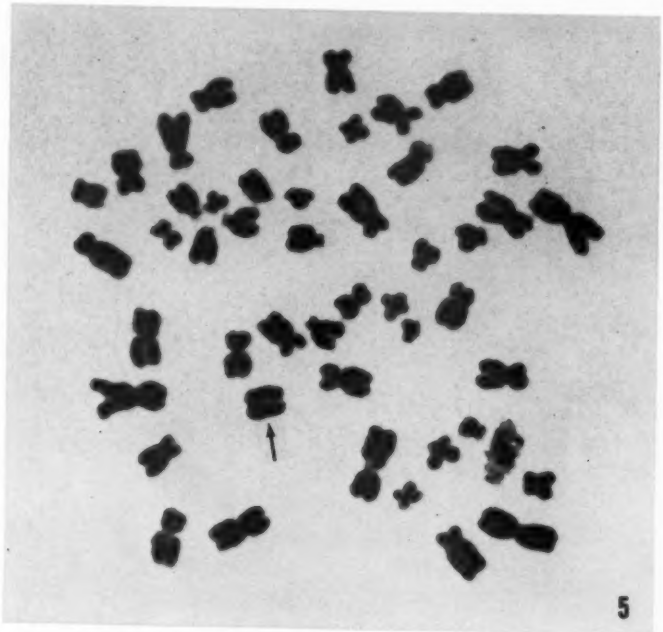
PLATE 13

FIGURE 5.—Metaphase from Normal case 2 (female), 47 chromosomes. *Note*, in addition to normal female complement, a large acentric or telocentric fragment (*arrow*). × 2,700

FIGURE 6.—Metaphase from Normal case 3 (male), 46 chromosomes. *Note* Y chromosome (*arrow*). *See* idiogram, figure 9. × 2,700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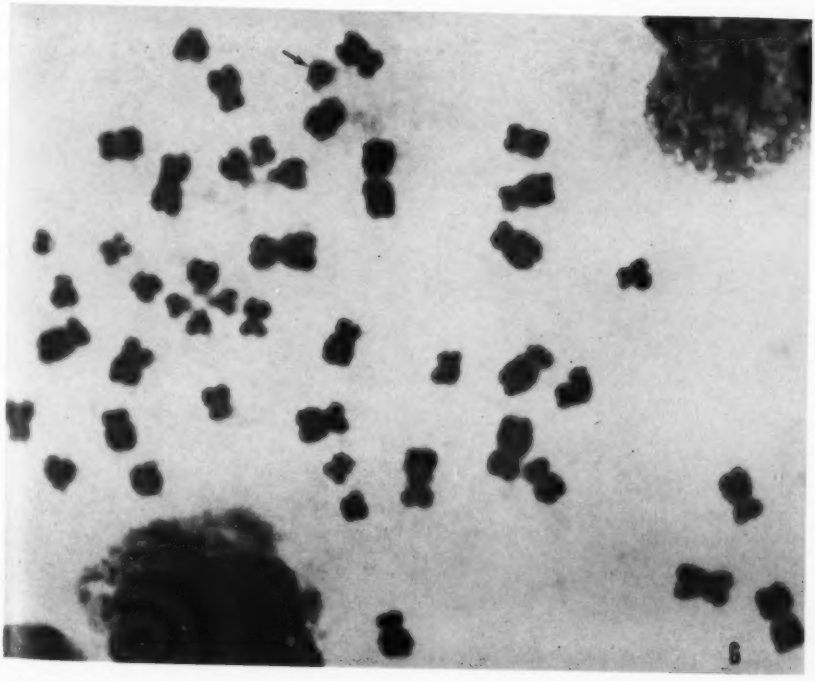
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PLATE 14

FIGURE 7.—Idiogram of metaphase shown in figure 3. × 2,700

FIGURE 8.—Idiogram of metaphase shown in figure 4. × 2,700

FIGURE 9.—Idiogram of metaphase shown in figure 6. × 2,700

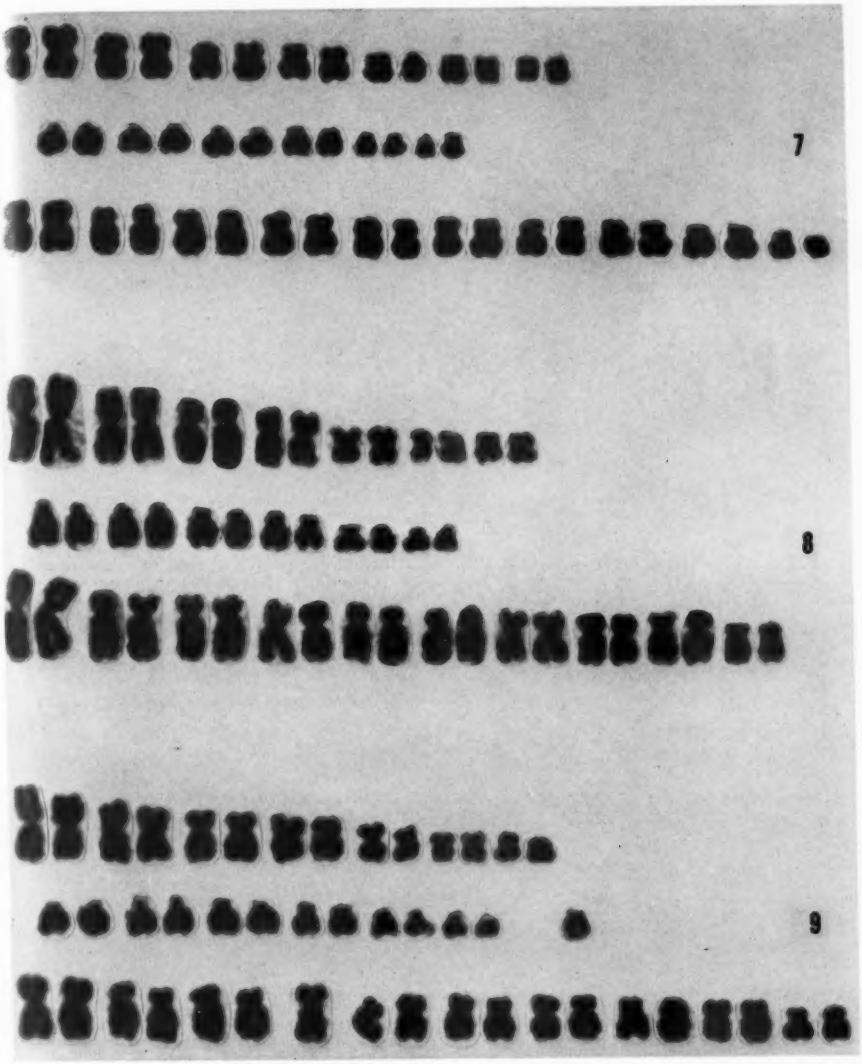
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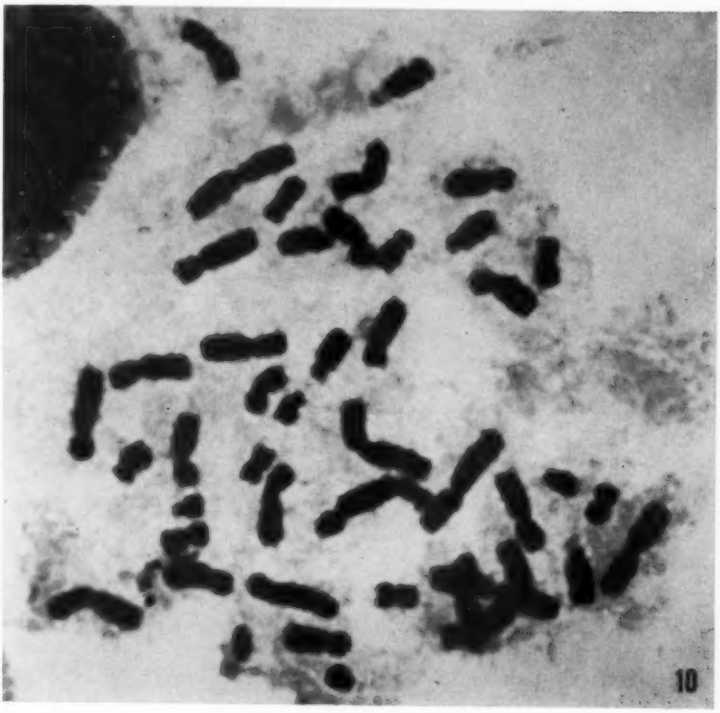
PLATE 15

FIGURE 10.—Metaphase from Leukemia case 1 (male), 46 chromosomes. *See* idiogram, figure 15. × 2,700

FIGURE 11.—Metaphase from Leukemia case 2 (female), 46 chromosomes. *See* idiogram, figure 16. × 2,700

FIGURE 12.—Metaphase from Leukemia case 3 (male), 46 chromosomes. *Note* minute chromosome (*arrow*). *See* idiogram, figure 17. All 3 metaphases analyzed from this case were similar. × 2,700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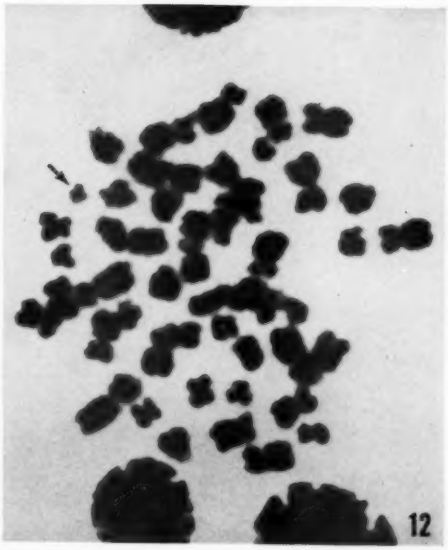
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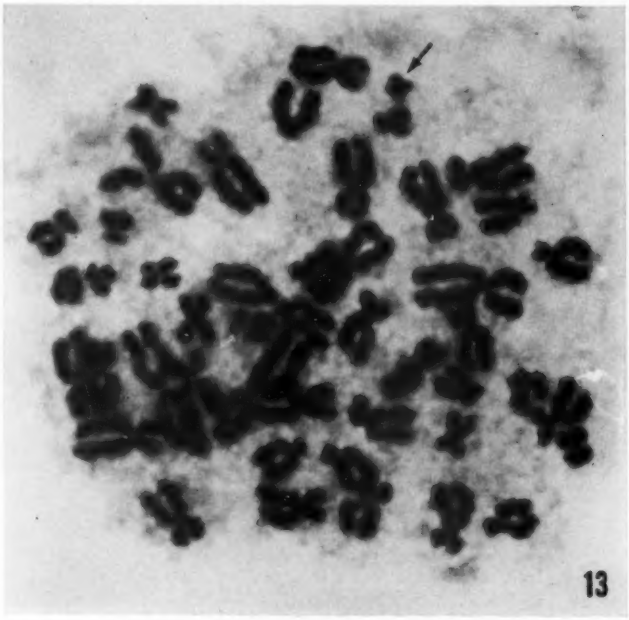
PLATE 16

FIGURE 13.—Metaphase from Leukemia case 4 (male), 46 chromosomes. *Note* minute chromosome (*arrow*). *See* idiogram, figure 18. × 2,700

FIGURE 14.—Another metaphase from Leukemia case 4, 46 chromosomes, no minute. *See* idiogram, figure 19. Of 5 metaphases that were analyzed, this is the only one with a normal male complement; all others were similar to figure 13. × 2,700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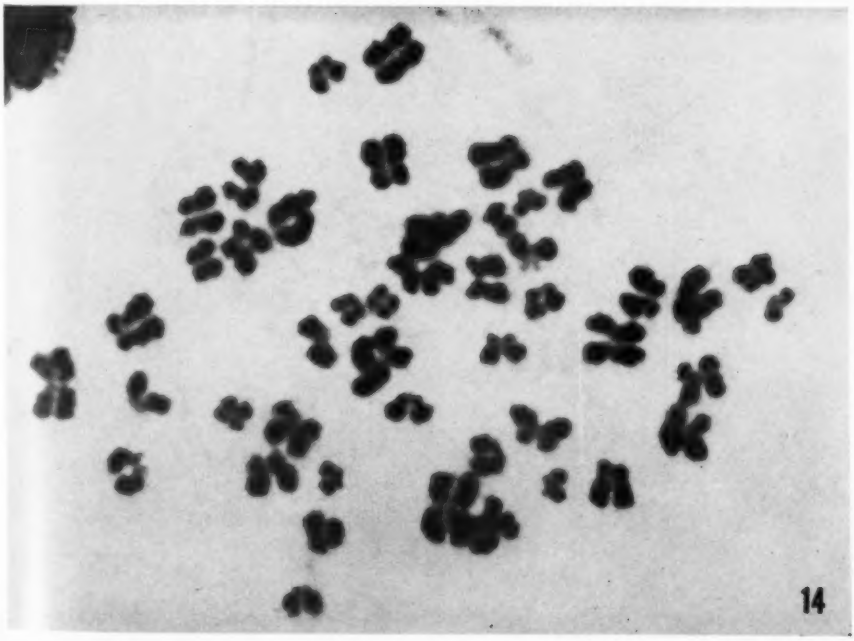
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PLATE 17

FIGURE 15.—Idiogram of metaphase shown in figure 10. × 2,700

FIGURE 16.—Idiogram of metaphase shown in figure 11. × 2,700

FIGURE 17.—Idiogram of metaphase shown in figure 12. × 2,700

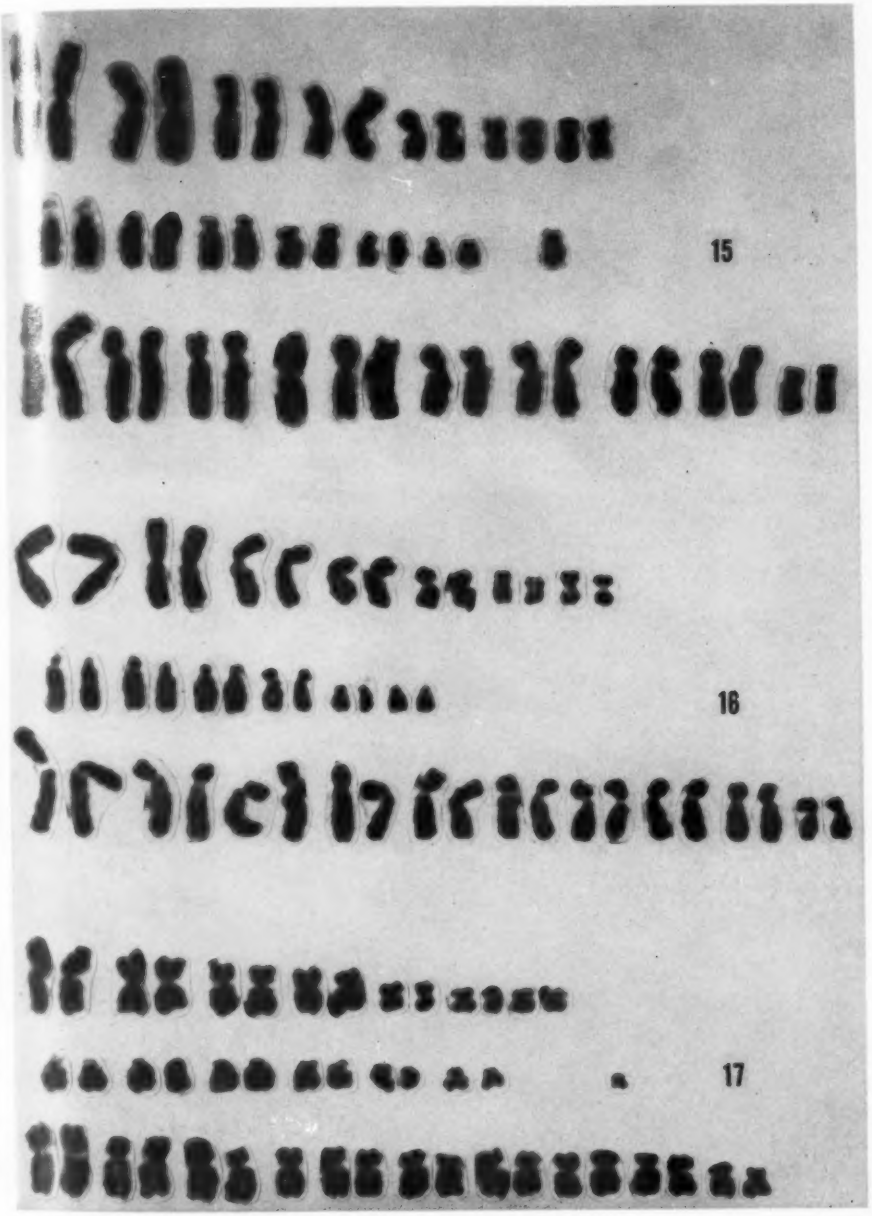
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PLATE 18

FIGURE 18.—Idiogram of metaphase shown in figure 13. × 2,700

FIGURE 19.—Idiogram of metaphase shown in figure 14. × 2,700

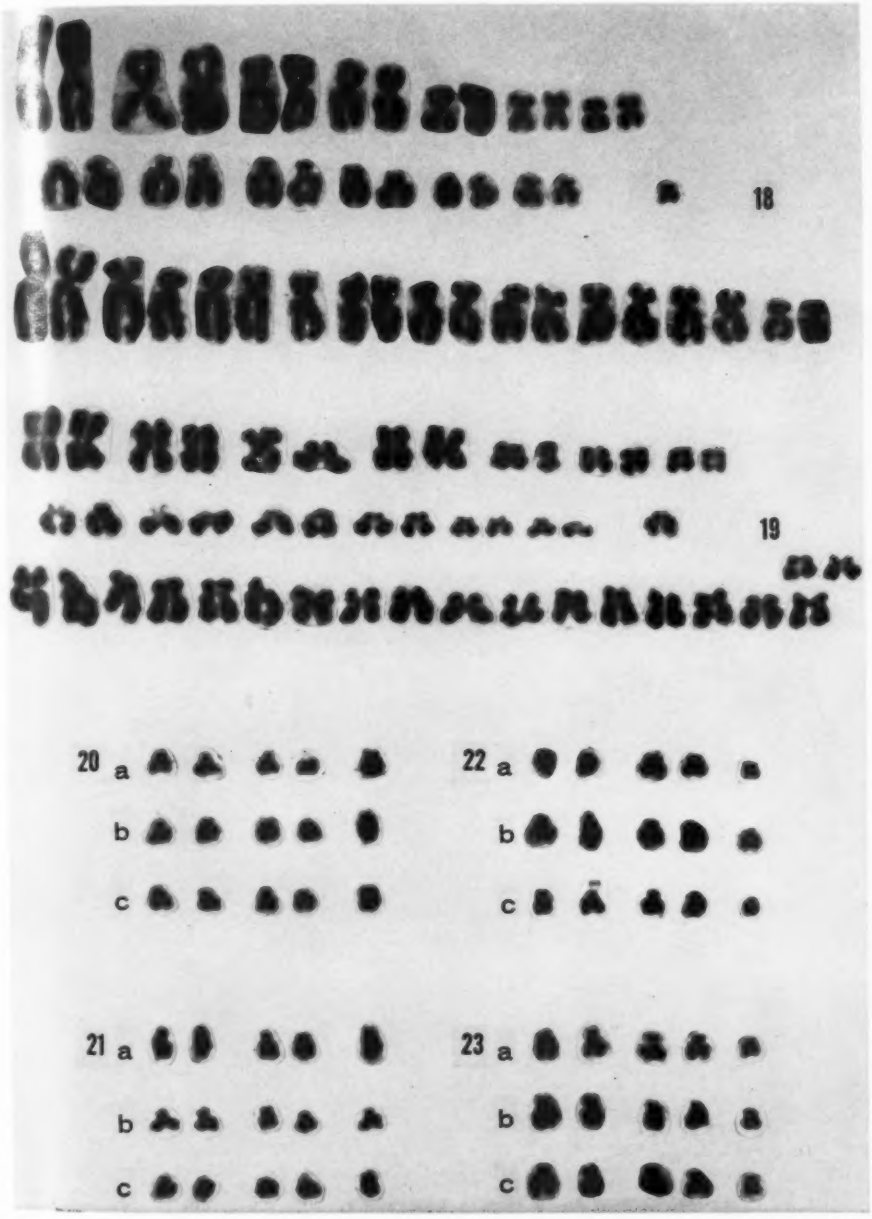
A comparison of the four smallest acrocentric autosomes and the Y chromosome from a normal male (fig. 20a–c) with the same chromosomes from Leukemia case 1 (fig. 21a–c) and with the same autosomes and the minute chromosome from Leukemia case 3 (fig. 22a–c) and Leukemia case 4 (fig. 23a–c). × 2,700

FIGURE 20.—*a:* Chromosomes from figures 6 and 9. *b, c:* Chromosomes from two other metaphases of Normal case 3.

FIGURE 21.—*a:* Chromosomes from figures 10 and 15. *b, c:* Chromosomes from two other metaphases of Leukemia case 1.

FIGURE 22.—*a:* Chromosomes from figures 12 and 17. *b, c:* Chromosomes from two other metaphases of Leukemia case 3.

FIGURE 23.—*a:* Chromosomes from figures 13 and 18. *b, c:* Chromosomes from two other metaphases of Leukemia case 4.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19
20 a
b
c
21 a
b
c
22 a
b
c
23 a
b
c

Transplantation Immunity Involving the *H-3* Locus: Graft Survival Times[1,2]

JAMES H. BERRIAN, *Ph.D., and* CHARLES F. McKHANN, *M.D.*,[3,4] *Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland*

SUMMARY

The behavior and survival times of skin homografts in coisogenic strains of mice differing by a single histocompatibility gene at the *H-3* locus were studied. Several methods for determining graft survival were compared. The range of first-set survival times in the strain combinations used were: C57BL/10Sc → C57BL/10-*H-3*^{b}*A*, 16 to 27 days; C57BL/10Sc → C57BL/10-*H-3*^{b}, 15 to 24 days; C57BL/10-*H-3*^{b}*A* → C57BL/10Sc 28 to >78 days; C57BL/10-*H-3*^{b} → C57BL/10Sc, 38 to >60 days. The homograft reactions resulting from weak genetic differences were not only slower to appear but also milder in degree than those resulting from *H-2* incompatibility. The "spread" of graft breakdown times around the median increased as the genetic difference between graft and host became less, which suggests that other factors than specific antigenicity of the grafted tissue influence the over-all reaction. At any given time, the basal cells of the epidermis of a graft were more greatly affected by immunity than more superficial cells. This supports the theory that graft destruction is the result of a cytotoxic reaction rather than ischemia after vascular stasis. Survival times of second-set grafts were more uniform; all were destroyed by the 8th day in the combination C57BL/10Sc → C57BL/10-*H-3*^{b}*A*, and by the 11th day in the reverse direction.—J. Nat. Cancer Inst. 25: 111–123, 1960.

THE ANTIGENS responsible for transplantation immunity in the mouse are products of genes at many different loci, genes that have been shown by Counce *et al.* (*1*) and by Barnes and Krohn (*2*) to vary greatly in their effects on the survival time of skin homografts. Strong responses commonly encountered among inbred strains of mice by virtue of *H-2* incompatibility have undoubtedly obscured recognition of the properties and behavior of many lesser antigens, so that it is now appropriate to question whether our present knowledge of these substances is representative. The variations may reflect differences in the quality and

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[2] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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[4] The authors wish to acknowledge the excellent technical assistance provided by Mr. H. L. Lemke and Mr. T. Standifer of the Naval Medical Research Institute.

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quantity of antigens elaborated by the genes, in their distribution and attachments in the tissues, and in other factors that could influence the development of immunity in the incompatible host. It has previously been observed (*3*) that certain antigens associated with genes of equivalent "strength"[5] could not be extracted by procedures that were effective for others (*4*), a phenomenon that has been attributed to molecular diversity far greater than the minimum required to account for differences in immunologic specificity. To explore this hypothesis further, a study was undertaken to compare the properties and behavior of transplantation antigens associated with genes of markedly different strength. The coisogenic trio of mouse strains developed by Snell (*5*) and employed by Counce *et al.* (*1*) to demonstrate variations in the strength of genes at the *H-2* and *H-3* loci was used for this work.

MATERIALS AND METHODS

The mice used throughout these experiments were stock derived from pedigreed pairs of siblings generously provided by Dr. George D. Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. For convenience, the strains are abbreviated in this paper as B, D, E, and G, for the lines C57BL/10Sc, C57BL/10-*H-2*^{d}, C57BL/10-*H-3*^{b}, and C57BL/10-*H-3*^{b}*A*, respectively, as shown in table 1. The derivation of these lines and their genetic composition have been discussed by Snell (*6*). In theory, the strains are coisogenic except for the alleles at the *H-2*, *H-3*, and agouti (*A*) loci, and possibly for genes closely linked with these alleles. In the following section some additional evidence is presented confirming the composition shown in table 1.

TABLE 1.—Mouse strains

Abbreviated designation	Strain	Differential alleles			
		H-2	*H-3*	Agouti (*A*)	Hair phenotype
B	C57BL/10Sc	*b*	*a*	*a*	Black
D	C57BL/10-*H-2*^{d}	*d*	*a*	*a*	Black
E	C57BL/10-*H-3*^{b}	*b*	*b*	*a*	Black
G	C57BL/10-*H-3*^{b}*A*	*b*	*b*	*A*	Agouti

Skin homografts were prepared and exchanged according to the method of Billingham and Medawar (*7*). Graft breakdown was determined and studied in 3 ways: (1) visual inspection of grafts from which the dressings

[5] "Strength" of histocompatibility genes refers to the activity of the corresponding antigens in promoting homograft immunity in incompatible recipients. Strong genes may be defined as those giving rise to early homograft rejection, weak ones permit longer survival. No connotation is implied with respect to the dominance or recessiveness of histocompatibility alleles. All known histocompatibility alleles are fully dominant.

were removed only at the time of inspection, (2) histologic examination of fixed and stained sections of grafts removed at appropriate intervals after grafting, and (3) periodic inspections of grafts from which the dressings were removed after an initial healing period of 12 days. It will be shown that for the determination of homograft survival times involving the weak *H-3* genetic differences the latter method is probably best suited.

In these experiments both males and females were used, but care was taken not to cross sex lines in exchanging grafts. Male recipients received only male homografts and female recipients only grafts from female donors, thereby avoiding sex-linked histocompatibility factors.

EXPERIMENTS

Number of Gene Differences Between Strains

The survival of parental-strain skin homografts on the F_2 recipients of 2 crosses (B × D) and (B × G) has thus far been determined for periods long enough to permit conclusions concerning the number of histocompatibility-gene differences between them. For this purpose, each of the F_2 recipients received grafts on opposite sides of the chest from both parental strains. In this test the expected number of permanently accepted grafts from either strain is $(3/4)^n$ where n is the number of differential genes. Of 52 F_2 recipients of B and D parental-strain grafts, 26 accepted both B and D grafts, 9 accepted only their B strain graft, and 16 accepted only their D strain graft. The observed deviation from a one-gene ratio (1:2:1) is not significant ($P = 0.4$) and the deviation from a two-gene ratio is highly significant ($P = < 0.01$). Of 50 F_2 recipients of their parental strain B and G grafts, 27 accepted both, 7 accepted only their B strain graft, and 14 accepted only their G strain graft. The observed deviation from a one-gene ratio is not significant ($P = 0.27$) and the deviation from a two-gene ratio is highly significant ($P = < 0.01$). The (B × D)F_2 recipients have been followed for over 200 days, and the (B × G) recipients for over 160 days. The results provide assurance that if there are other histocompatibility differences between the strains than the ones indicated in table 1, these are much weaker than the *H-3* genes, and are, therefore, inconsequential by comparison.

Additional confirmation that there are no histocompatibility differences of importance between the E and G strains is provided by the observation that grafts exchanged between these strains are fully surviving at well over 225 days. Isogenicity within each of the lines seems assured by the fact that isografts exchanged between individuals of the same line have each been followed for 200 days without evidence of incompatibility. Our data on these strains, with skin homografts as the criterion, fail to disclose any deviation from the genotypes and degree of coisogenicity that they should possess according to Snell's theoretical expectations.

Gross Characteristics of Graft Breakdown

By definition, weak histocompatibility differences allow a homograft to survive longer than do strong differences. A quantitative description of this in terms of graft survival times will be given. Qualitative differences were also found. When grafts were exchanged in either direction between strains B and G, many of the grafts survived long enough to regenerate hair. This growth was irregular in the series of grafts, but without exception, it was *less* at any given time than that seen in isograft controls. With grafts of B to G, where graft survival time was shorter, few grafts showed more than early hair regeneration prior to their breakdown. Conversely, when the donor and recipient were reversed, most grafts evidenced some new hair but none had the luxuriant growths characteristic of isografts of 30 to 40 days' duration. It was observed that the growth of hair in an isograft frequently exceeded that of the host for many months after grafting, irrespective of normal cyclic variations in the growth of the recipient's coat.

In grafts that were left exposed to the air after the dressing was removed at 12 days, the earliest signs of impending breakdown were loss of any existing hair and scaliness of the epithelium. This was followed within a few days by apparent drying and eschar formation. These "scabs" either appeared singly and grew larger or there were several that grew confluent with time. Eventually the eschar covered the entire area of the graft, its surface becoming concave as it dried out and the wound contracted. In the final stage the host rejected the remnant of the graft and the remaining small wound was obliterated by scar tissue. Outwardly, the graft rejection appeared to be a mild reaction in that there was less evidence of inflammation or of the subepithelial hemorrhages usually seen in strong tissue immunity. The duration of the course of destruction of individual grafts was prolonged, in that the interval from the time of the earliest signs of epithelial distress to the time of complete replacement of the graft surface by eschar was several days and often exceeded a week. In *H-2* incompatibilities this period rarely exceeds 2 days.

Histologic Studies of Graft Breakdown

Grafts in the combination B to G were removed between 16 and 22 days for histologic examination. Even at the height of graft destruction the cellular reaction in the graft bed was less than that associated with transplantation across the *H-2* locus (B → D). Figures 1 and 2 show typical sections of 2 first-set grafts, both evidencing complete destruction of the epithelium. Figure 1, at 10 days, involves a strong genetic difference at *H-2*, and figure 2, at 20 days, involves a weak difference at *H-3*. The first section is typical of the violent reaction seen in *H-2* grafts. The second shows not only less reaction than the first but also represents the greatest reaction seen in any of the 36 *H-3* grafts examined histologically.

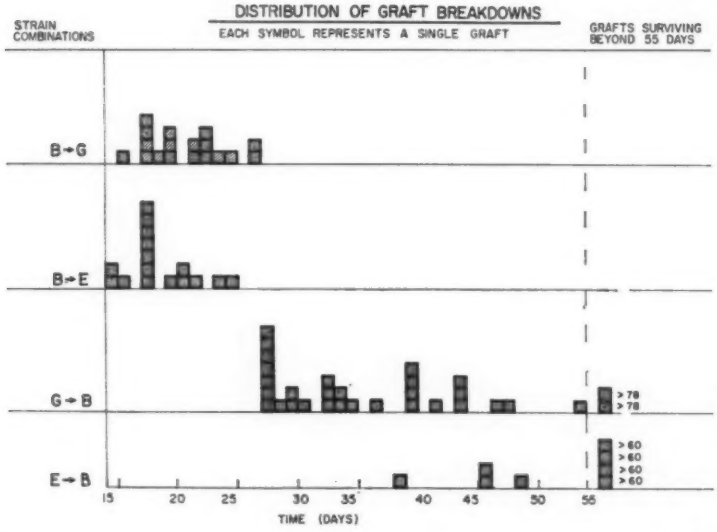
The variability of the reaction is exemplified by the following. Figures 3 and 4 show 2 grafts at 16 days, both involving only weak genetic differences at *H-3*. In one there is marked early hair growth and normal-appearing surface epithelium. In the other, hair regeneration is nil, and the epithelium has already been destroyed by a cellular reaction that is mild when compared with that of the graft shown in figure 2. In some sections the hair follicles and surface epithelium could be clearly seen as targets for the lymphocytic cells (fig. 5); in others the remnants of such follicles were in continuity with the few remaining cells of surface epithelium (fig. 6). Vascular dilatation, hemorrhages, and extravasations of red blood cells into the grafts were less pronounced than in *H-2*.

In this same series of grafts, an attempt was made to determine the median survival time (MST) by sequential sampling [*cf.* Billingham (*8*)]. Grafts were removed in sets of 6 at 16, 18, 20, 22, and 24 days, exposure having been prevented up to that time by their dressings. Visual inspections were equivocal. Representative sections from areas through the grafts were examined histologically. Marked variability was evidenced in the entire series and even existed between different areas of the same graft. This was particularly true of the basal layer of epithelial cells. Areas of pyknotic cells, with or without associated host-cell infiltration, were interspersed with areas of normal-appearing cells. In virtually all, the basal cells appeared to have undergone more extensive degeneration than the overlying epithelium. An additional confusing factor was the apparent improvement shown in grafts removed at 20, 22, and 24 days. The "epithelial recovery" was maximal at the periphery of the graft and minimal or nonexistent in the center. Later grafts (22 and 24 days) also contained proliferative epithelium in the center. Although the possibility could not be ruled out that the grafts were re-epithelialized by cells emanating from surviving follicles (*see* fig. 6), it is more likely that this represents ingrowth of the host epithelium and timely replacement of that destroyed by immunity. Determination of the MST by this method was not practical because of the ambiguity of epithelial replacement.

Time of Graft Breakdown

In another series of mice the dressings were removed at 12 days and periodic inspections were made thereafter. Grafts were exchanged in both directions between strains B and G and B and E. The criterion of graft breakdown was the complete replacement of previously normal epithelium by a dry eschar. The data are shown in text-figure 1. In the 4 strain combinations used, there were 2—possibly 3—different rates of immune response. B to G and B to E were both relatively rapid (MST = 21 ± 0.8 and 19 ± 0.8 days, respectively). G to B was slower and E to B, within the limitations of a small sample, appeared slower still. A marked difference was noted in the survival time of grafts in reciprocal com-

binations: B to G survived from 16 to 27 days, but in G to B survival ranged from 28 to 87 days. In those grafts of unexpectedly long survival, careful inspections were made to prevent mistaking insidious overgrowth of host epithelium for true survival. In addition, in this particular combination, there is a difference of hair color that makes identification of the graft more certain.



TEXT-FIGURE 1.—Distribution of graft breakdown times in strains B → G, B → E, G → B, and E → B.

Time of Second-Set Breakdown

After immunization with skin grafts, within a few days the recipient animals were regrafted from the same donor strain. Results are given in table 2. In both directions of the *H-3* incompatibility it was found that about half the grafts were completely broken down by 6 days and all by

TABLE 2.—Survival times of second-set grafts

Strain combinations	Interval between graftings (*days*)	Inspection (*day*)	Graft scores (*estimated % surviving epithelium*)
B⟶G	25–50	6	100−, 100−, 90, 90, 75, 75, 0, 0; 100, 75, 50, 25, 25, 0, 0, 0; 50, 25, 25, 0, 0, 0, 0
		8	0, 0, 0, 0, 0, 0, 0, 0; 0, 0, 0, 0, 0, 0, 0, 0; 0, 0, 0, 0, 0, 0, 0, 0, 0
B⟶G	145	8	0, 0, 0
G⟶B	43	8	100, 100−, 100−, 100−, 75
		11	90, 0, 0, 0, 0
		13	25, 0, 0, 0, 0
		15	0, 0, 0, 0, 0

8 days. Nonhealing grafts of the types originally described by Billingham *et al.* (*9*) were not seen. No attempt was made to determine the duration of the immunity, though it retained full activity at 145 days.

DISCUSSION

The findings reported here do not differ from those of Counce *et al.* (*1*) in that histocompatibility loci and even single alleles of a gene locus have characteristic strengths. To get consistent endpoints in determining the survival times of grafts, it was necessary to leave them exposed and inspect them periodically after an initial healing period. The replacement of the graft epithelium with a dry eschar was easily identified even in grafts of prolonged survival. It may be that the presence of a dressing during the period of epithelial breakdown afforded protection to the graft and allowed the insidious replacement of nonviable cells with ingrowing host epithelium.

Generally, in strain combinations permitting more prolonged graft survival, the "spread" of survival times around the median is greater. In *H-2* incompatibilities this spread around the MST rarely exceeds 2 days in either direction, a total of 4 days. In the *H-3* combination of B to G this spread encompasses a total of 12 days, and in G to B it exceeds 27 days. It is not unexpected that when only weak histocompatibility differences are involved, factors in addition to the specific antigenicity of the grafted tissue come into prominence. These could include biologic variation in the responsiveness of the host (genetic variation not associated with histocompatibility genes), specific protection of the graft (tolerance or enhancement), or some form of adaptation of the graft itself (*10*).

Histologic sections of several grafts suggested that "activity" of the skin and, particularly, hair regeneration were associated with temporarily enhanced survival. Without direct evidence to the contrary, it is possible that the far greater number of cells present in grafts of "active" skin are able to tolerate a limited amount of immunologic opposition without ill effects; as more antibody becomes available the cells might then succumb.

The finding that basal cells consistently appeared more vulnerable to immunity than overlying epithelial cells supports the theory that homograft destruction is the result of a "toxin" or toxic reaction diffusing from deep in the graft and not simply the result of vascular stasis and ischemia, which would be expected to affect more superficial cells first by depriving them of needed metabolites.

Since second-set survival times more often are used as criteria in experimental designs than first-set survival, it is, perhaps, fortunate that they do not show the variability associated with the latter.

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PLATES

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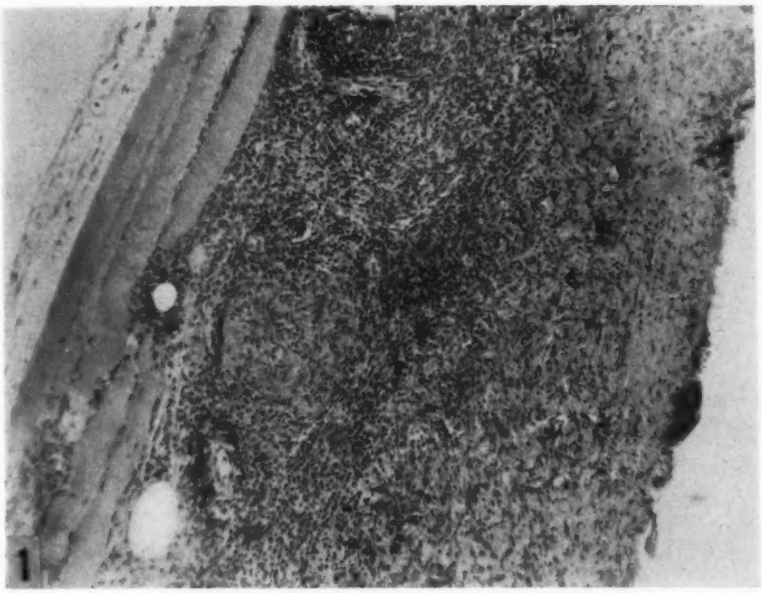
PLATE 19

FIGURE 1.—*H-2* immunity at 10 days showing typical strong cell reaction, epithelium destroyed. × 118

FIGURE 2.—*H-3* immunity at 20 days showing lesser lymphoid-cell reaction of weak immunity, epithelium destroyed. × 118



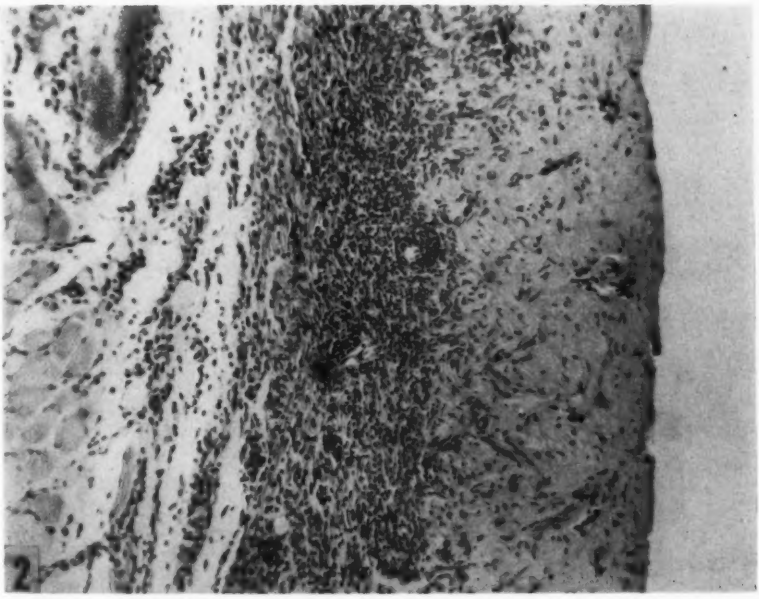
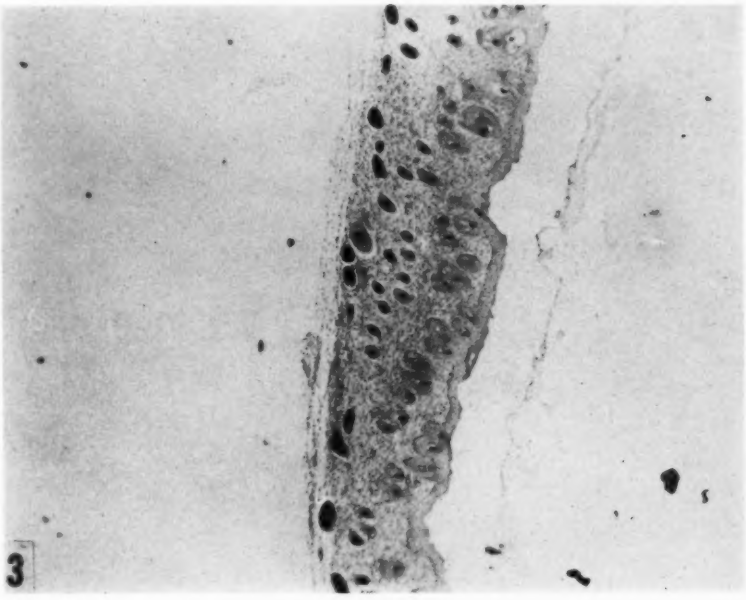


PLATE 20

FIGURE 3.—*H-3* immunity at 16 days. Graft shows extensive hair regeneration, moderate lymphoid infiltration, but fully surviving, hyperplastic epithelium. × 39

FIGURE 4.—*H-3* immunity at 16 days. Graft shows no hair regeneration, minimal cellular infiltration but far-advanced destruction of surface epithelium. × 118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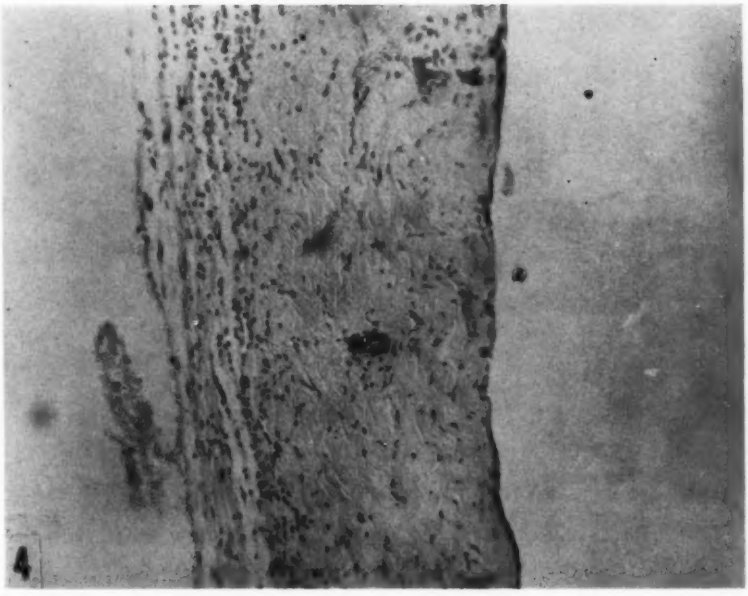
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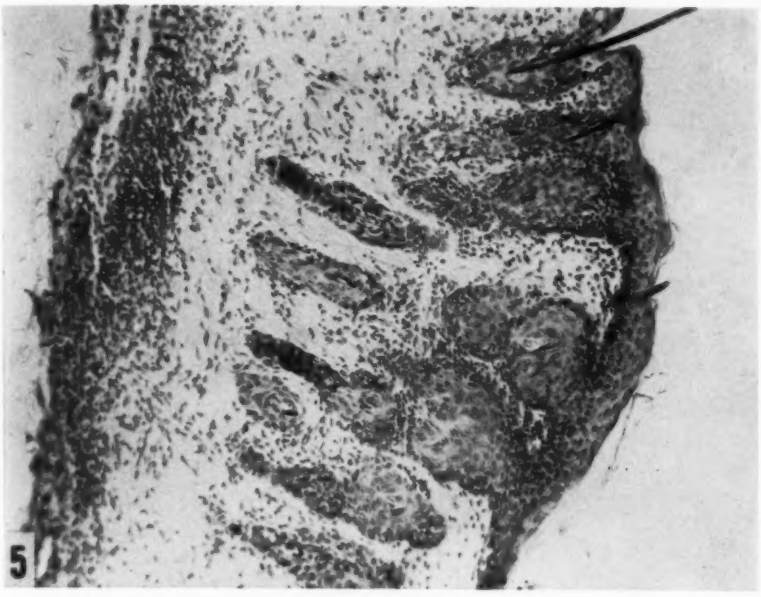
PLATE 21

FIGURE 5.—*H-3* graft at 20 days showing lymphoid cells in contact with hair follicles and surface epithelium. Epithelium intact. × 118

FIGURE 6.—*H-3* graft at 20 days showing greatly thinned-out surface epithelium in contact with flattened, degenerating hair follicle. × 118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Method of Biological Assay of Compounds Effective in the Treatment of IRC 741 Leukemia in Fischer Rats [1,2]

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SUMMARY

An assay system with a wide range for testing carcinolytic compounds in the development of the transplantable acute leukemia IRC 741 in Fischer rats is described. By initiating treatment at intervals of 1, 2, 4, 6, 8, and 10 days after transplantation, it is possible to show differences in response to compounds which are not discernible when testing is limited only to early and established tumors. The method is presented as a biological tool for demonstrating differences in response that may result from modifications of active antileukemic compounds. The common denominator for comparison may be dosage and/or treatment schedule, percent cures on day 1, or 2, vehicle, or route of administration of the compound. The method may also be used in selecting an optimum regimen of treatment for a compound. The application of this procedure to other tumor systems has been suggested.—J. Nat. Cancer Inst. 25: 125–131, 1960.

A BIOLOGICAL assay system for testing carcinolytic compounds was developed after it was observed that the reduction in effectiveness of compounds tested at frequent intervals in the course of development of IRC 741 leukemia in Fischer rats was not a uniform and gradual decline. Data obtained by testing Nitromin, Elderfield mustard, Tetramin, tris(1-aziridinyl)-phosphine sulfide (TSPA), mannitol mustard, nitrogen mustard, amethopterin, and urethan by the delayed-treatment method are presented to support this observation. Response to treatment with 2,5-bis(1-aziridinyl)-3,6-*dipropoxy-p*-benzoquinone (E-39) and 2,5-bis(1-aziridinyl)-3,6-*bis(2-methoxyethoxy)-p*-benzoquinone (A-139) suggests that the assay system is worthy of further investigation.

The assay system was also used to compare dose response and the effect of differences in treatment schedule for chlorambucil. Endoxan was tested in sesame oil and saline to compare the effect of a difference in the vehicle.

All the compounds tested, except amethopterin and urethan, were alkylating agents. The assay system utilized compounds that were

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readily available. They were obtained from the Cancer Chemotherapy National Service Center, of the National Cancer Institute, through the Cancer Research Division of the Department of Medicine, University of Miami, School of Medicine.

MATERIALS AND METHODS

The tumor IRC 741, a transplantable acute leukemia used in testing the compounds, originated in a Fischer line 344 rat. This tumor was described by Dunning and Curtis (*1*) in 1957. Jones *et al.* (*2*) in 1958 reported the usefulness of this transplantable tumor for quantitative pharmacological studies of cancer chemotherapeutic agents.

After subcutaneous implantation the tumor was palpable in 6 to 8 days and by the 10th day was well established. The mean survival time of 760 untreated rats after transplantation was 14.2 days with a range from 12 to 18 days.

The inbred Fischer line 344 rats were bred in this laboratory and used when about 6 weeks old. At this time they weighed 60 to 80 gm.

Tissue for transplantation was removed aseptically from the subcutaneous growth of a donor rat and placed in a sterile petri dish containing 5 to 10 cc. of normal saline. Fragments of tumor tissue, measuring 1.5 to 2.0 $mm.^3$, were implanted subcutaneously by trocar into the right flank of each recipient.

For each test 60 or 70 rats bearing tumor implants were divided into groups of 10. One group served as a control. Treatment of the first group of rats was initiated 24 hours after transplantation and the second group after 48 hours. Treatment of the remaining groups was delayed 4, 6, 8, and 10 days. All compounds were dissolved or suspended in saline and were injected intraperitoneally. Variations from this procedure have been specified.

Treated rats that survived for 90 days without tumors were considered "cured" and were challenged with a second implant of the tumor. All died with tumors within 12 to 19 days after reinoculation. Rats that died before treatment was completed or died without a tumor 5 days after treatment was terminated were considered toxic deaths. Data for treated rats that died, with or without tumors, after the controls died were combined in calculating mean survival and percent increase in survival.

The mean percent increase (MPI) in survival of treated (T) rats beyond the average survival of the control (C) rats was calculated by the formula $\frac{T-C}{C} \times 100 = MPI.$

RESULTS

The results of subjecting 10 compounds to this biological assay system are given in table 1. These compounds were selected to show that the

TABLE 1.—Response of IRC 741 leukemia in Fischer line 344 rats to active chemotherapeutic agents when treatment was initiated 1, 2, 4, 6, 8, and 10 days after transplantation

	Compound	Dose (*mg./kg.*)	Treatment schedule	Delay (*days after transplantation*)	Ratio (*No. cured/No. treated*)	Survival of rats not cured (*days*) Mean	Range	Toxic deaths of rats (*percent*)
1	Nitromin	8.0	q4d × 4	Control	0/10	13.7	13–14	0
				1	8/10	29.0	23–35	0
				2	8/10	27.5	24–31	0
				4	8/10	29.5	29–30	0
				6	0/10	31.6	28–41	0
				8	0/10	31.4	28–34	20
				10	0/10	29.2	28–32	20
2	Elderfield mustard	1.0	qd × 12	Control	0/10	13.5	12–15	0
				1	8/10	54.5	41–68	0
				2	7/10	32.3	27–36	0
				4	1/10	39.3	23–68	0
				6	0/10	26.7	22–29	0
				8	0/10	27.4	26–30	0
				10	0/10	27.9	27–29	0
3	Tetramin	5.0	qd × 12	Control	0/10	13.4	12–15	0
				1	8/10	42.0	28–56	0
				2	5/10	51.2	29–80	0
				4	5/10	30.6	26–34	0
				6	1/10	29.4	26–33	0
				8	0/10	31.4	30–34	0
				10	0/10	31.4	28–35	0
4	TSPA	1.5	q2d × 7	Control	0/10	14.0	12–15	0
				2	4/10	40.8	25–63	0
				4	1/10	30.0	24–40	0
				6	0/10	28.1	24–32	0
				8	0/10	28.6	27–30	0
				10	0/10	29.6	26–32	0
5	Mannitol mustard	3.0	qd × 13	Control	0/10	14.0	13–16	0
				2	4/10	31.6	29–37	0
				4	0/10	31.6	28–35	0
				6	0/10	30.7	29–36	0
				8	0/10	31.5	29–36	0
				10	0/10	32.7	30–36	0
6	Nitrogen mustard	0.45	q4d × 4	Control	0/10	14.1	13–16	0
				1	0/10	31.5	28–36	0
				2	0/10	30.2	28–36	0
				4	0/10	26.3	26–33	0
				6	0/10	26.9	25–28	0
				8	0/10	24.6	25–29	0
				10	0/10	25.0	21–27	0
7	Amethopterin	3.0	q4d × 4	Control	0/10	13.7	13–14	0
				1	0/10	29.4	28–31	0
				2	0/10	29.5	23–33	0
				4	0/10	30.8	28–33	0
				6	0/10	27.4	25–32	0
				8	0/9	30.1	28–33	10
				10	0/10	28.6	25–32	50

TABLE 1.—Response of IRC 741 leukemia in Fischer line 344 rats to active chemotherapeutic agents when treatment was initiated 1, 2, 4, 6, 8, and 10 days after transplantation—Continued

	Compound	Dose (*mg./kg.*)	Treatment schedule	Delay (*days after transplantation*)	Ratio (*No. cured/No. treated*)	Survival of rats not cured (*days*) Mean	Range	Toxic deaths of rats (*percent*)
8	Urethan	300	qd × 19	Control	0/10	13.6	12–14	0
			× 19	2	0/10	24.1	22–26	0
			× 19	4	0/10	25.0	24–26	0
			× 17	6	0/10	24.7	24–26	0
			× 15	8	0/10	24.6	23–26	0
			× 12	10	0/10	24.6	23–25	0
9	E-39	1.0	q2d × 7	Control	0/10	14.8	14–16	0
				2	9/10	54	54	0
				4	5/10	34.2	26–54	0
				6	1/10	33.6	27–47	0
				8	0/10	31.5	29–34	0
				10	0/10	32.0	30–33	0
10	A-139	0.4	qd × 12	Control	0/10	12.8	12–14	0
				2	9/10	64	64	0
				4	0/10	32.4	22–37	0
				6	0/10	28.7	26–38	0
				8	0/10	29.5	28–31	0
				10	0/10	29.0	28–31	0

activity of a compound cannot be predicted by testing only the response of early and established tumors. Urethan and amethopterin were added to the list to emphasize the importance of "noncurative compounds" in the treatment of established tumors.

Table 2 compares biological responses to compounds that were similar when treatment was initiated 24 or 48 hours after tumor transplantation and showed differences at later intervals of testing. A uniform dosage or treatment schedule did not seem necessary because comparison was based on uniform responses to treatment on days 1 or 2.

Comparison of Nitromin, Elderfield mustard, and Tetramin was based on 80 percent "cures" on day 1. Significant differences appeared when treatment was delayed 2 days or more. Nitromin produced 80 percent cures on days 2 and 4. The increase in survival for rats that were not "cured" was nearly the same for all the intervals tested. In response to treatment with Elderfield mustard the number cured was reduced from 80 percent on day 1 to 70 percent on day 2. The survival of rats that were not "cured" was reduced from 303 to 139 percent, followed on day 4 by only 10 percent "cures" and 191 percent increase in survival. On days 6 to 10 survival was about 100 percent longer than controls. Treatment with Tetramin on day 2 produced 50 percent "cures," but survival of rats that were not "cured" increased from 213 to 282 percent. On day 4, 50 percent "cures" were associated with 119 percent increase in survival, and the increase in survival was 134 percent on days 8 and 10.

TABLE 2.—Response of IRC 741 to treatment with different compounds—comparison of compounds based on similar response at first interval of testing

Compound	Dose (*mg./kg.*)	Treatment schedule	Day treatment initiated: (*percent cured/percent increase survival*) 1	2	4	6	8	10
Nitromin	8.0	q4d × 4	80/112	80/101	80/115	0/130	0/130	0/112
Elderfield mustard	1.0	qd × 12	80/303	70/139	10/191	0/98	0/103	0/107
Tetramin	5.0	qd × 12	80/213	50/282	50/128	10/119	0/134	0/134
TSPA	1.5	q2d × 7	—	40/191	10/114	0/100	0/104	0/111
Mannitol mustard	3.0	qd × 13	—	40/126	0/126	0/119	0/125	0/133
Nitrogen mustard	0.45	q4d × 4	0/123	0/114	0/87	0/90	0/74	0/77
Amethopterin	3.0	q4d × 4	0/115	0/115	0/124	0/100	0/111	0/109
E-39	1.0	q2d × 7	—	90/265	50/131	10/127	0/113	0/116
A-139	0.4	qd × 12	—	90/400	0/153	0/124	0/130	0/127

Comparison of TSPA and mannitol mustard was based on 40 percent "cures" when treatment was started on day 2. The increase in survival for rats that were not cured was 191 percent in response to treatment with TSPA compared to 126 percent with mannitol mustard. On day 4 TSPA produced 10 percent "cures," but survival was reduced from 191 to 114 percent. Mannitol mustard did not produce "cures" on day 4, but there was no change in the mean survival for rats that were not "cured." From day 6 to day 10 mannitol mustard produced 20 percent longer survival than was observed for rats treated with TSPA.

Nitrogen mustard and amethopterin were compared to show that the procedure can be used to demonstrate differences in compounds that are not curative. Both compounds more than doubled the life of the rats when treatment was started on day 1, and this was not appreciably altered on day 2. Survival of the rats treated with nitrogen mustard was reduced from 114 percent on day 2 to 87 percent on day 4. This change of 27 percent was significant by the t test ($t_{20} = 4.43$). A similar change occurred in response to treatment with amethopterin, but it appeared between day 4 and day 6 ($t_{20} = 4.00$).

In testing chlorambucil at 2 dose levels (table 3, expt. 1), the higher dose produced cures on day 2. The activity was reduced at later intervals. The lower dose, which did not produce cures at any time, showed significant improvement in effectiveness from day 2 to day 8 ($t_{20} = 4.51$). Dose-response tests with chlorambucil, not included in this report, confirmed the results presented here. Treatment with Endoxan (table 3, expt. 2) in oil and in saline showed differences on days 4, 8, and 10 that could not be predicted from results obtained on days 1 and 6.

TABLE 3.—Dose response of IRC 741 to treatment with chlorambucil and comparison of response to Endoxan carried in sesame oil and in saline, tested by the delayed-treatment method

Compound	Dose	Treatment	Delay (*days after transplantation*)	Ratio (*No. cured/No. treated*)	Survival of rats not cured (*days*)		Increase in survival (*percent*)	Toxic deaths of rats (*percent*)
					Mean	Range		
Chlorambucil	5.0	q2d × 8	Control	0/10	14.5	13–17	0	0
			2	6/10	33.0	21–54	128	0
			4	0/10	40.3	25–80	178	0
			6	0/10	27.6	26–30	90	0
			8	0/10	29.2	27–31	101	0
			10	0/ 9	30.2	29–33	108	0
	2.5	qd × 13	Control	0/10	13.3	12–14	0	0
			2	0/10	23.7	23–26	78	0
			4	0/ 9	25.1	24–26	88	0
			6	0/10	25.5	24–26	92	0
			8	0/10	27.4	25–28	106	0
			10	0/10	27.1	25–29	104	0
Endoxan Oil, s.c.	20.0	q4d × 4	Control	0/10	13.5	12–16	0	0
			2	4/10	45.0	28–72	233	10
			4	4/10	42.8	26–75	217	10
			6	2/10	46.3	25–70	243	0
			8	0/10	39.2	32–47	190	0
			10	0/10	37.5	33–43	178	0
Saline, s.c.	20.0	q4d × 4	Control	0/10	12.4	12–13	0	0
			2	4/10	27.5	20–47	122	20
			4	7/10	31.3	30–33	152	0
			6	3/10	45.0	28–83	263	0
			8	0/10	48.3	32–56	289	30
			10	0/10	41.7	28–82	236	0

DISCUSSION

In the search for effective chemotherapeutic agents for the treatment of cancer many satisfactory primary and secondary evaluation methods have been devised (*3–6*). The purpose of these screening methods was to detect active compounds and to formulate the most effective regimen of treatment. The assay system presented in this report is suggested as a tool for subjecting positive compounds to biological analysis by comparing the effectiveness of a compound during the course of the disease. However the test may be useful in evaluating the comparative effectiveness of different compounds.

Table 2 was prepared to compare compounds that showed a similar response on day 1 or day 2. This leads to the suggestion that the biological assay method may be used to reflect molecular modifications of active compounds when dose-response methods of testing fail to show differences. The only compounds on hand to illustrate this point are 2,5-bis(1-aziridinyl)-3,6-*dipropoxy-p*-benzoquinone [(E-39), table 1, #9] and 2,5-bis(1-aziridinyl)-3,6-*bis*(*2-methoxyethoxy*)-*p*-benzoquinone [(A-139),

table 1, #10]. Ninety percent "cures" were obtained with treatment by both compounds on day 2. On day 4, E-39 produced 50 percent "cures"; none were "cured" with A-139 on day 4. If these 2 compounds had been tested on day 1 and day 7, possibly no difference would have been observed.

The response to chlorambucil was unexpected and is difficult to explain. There is no question about increased effectiveness of the treatment that seems to be associated with the development of the disease when the noncurative dose was used. When the mean survival for the 2 groups is compared, the higher dose is found more effective on days 2 and 4. The lower dose, however, was as effective as the higher dose on days 6, 8, and 10. Elucidation of the response to treatment with the low dose may be useful in chemotherapy studies.

The tests with chlorambucil and Endoxan show that dose, treatment schedule, route of administration, and vehicle may be useful variables for demonstrating differences in response to compounds that are not shown by other methods.

This procedure of testing compounds may be modified to serve specific needs:

1. The period of observation may be reduced from 90 to 50 days or less.
2. The intervals tested may be extended to only the 7th day after transplantation instead of the 10th day, since most of the significant differences appeared by the 6th day after transplantation.
3. Leukocyte count as well as survival time may be used as a basis of comparison; the size of the tumor may be used when effectiveness of a compound is measured by its capacity to reduce the size of the subcutaneous growth.
4. The mouse leukemia L1210 or any other transplantable tumor may be used in place of IRC 741, allowing intervals of delay appropriate to the degree of malignancy of the tumor.

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Evaluation of 5-Fluorouracil in the Treatment of Cancer [1]

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SUMMARY

5-Fluorouracil (5-FU) was used in 60 patients with advanced cancer classified as originating in 21 different sites. One case of Letterer-Siwe disease was treated. In 12 patients with advanced carcinoma, objective regression of tumor growth was noted (19.6%). The longest remissions occurred in cancer of the breast and uterus. Thirty-one patients (50.8%) had some degree of subjective relief. Toxicity was severe in most cases and fatal in 11 (18.0%), though the fatalities occurred usually in markedly debilitated patients with extensive cancer. The antineoplastic effect of this drug is of interest because it will produce remissions after other methods of treatment have failed. Perhaps related drugs will prove less toxic and more effective.—J. Nat. Cancer Inst. 25: 133–140, 1960.

5-FLUOROURACIL (5-FU),[3] developed by Heidelberger and collaborators, has been studied extensively in a variety of animal tumors (*1*, *2*). The use of this material in the treatment of human cancer has been the subject of 3 fairly comprehensive reports by Curreri, Ansfield, and their associates (*3*, *4*), and Gold *et al.* (*5*); a number of other investigators have reported its effect in small series of cases.[4] Ansfield and Curreri have found this drug most effective in treatment of carcinoma of the breast, ovary, large intestine, and rectum, and in hepatomas, and have described its toxicity. They state that the drug must be used with great care, especially when patients have had previous intensive roentgen-ray therapy of large flat bones such as the pelvis.

The present paper reports experience with this chemotherapeutic agent at the Albany Hospital and at the Albany Veterans Hospital. In the early part of this study, a number of patients with advanced cancer who were treated would not now be considered suitable for treatment.

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[2] The authors wish to acknowledge the cooperation of Dr. Robert V. Schatken of the Delaware Valley Hospital, Walton, N.Y.
[3] Supplied by Hoffmann-La Roche, Inc., Nutley, N.J., through the courtesy of Dr. M. J. Schiffrin.
[4] A brochure of other literature pertinent to 5-fluorouracil is available from Hoffmann-La Roche, Inc.

METHODS

Patients with advanced cancer were selected for treatment only if symptomatic and more conventional means of therapy had failed, and if they had either visible, radiographic, or palpably measurable lesions; hemograms and roentgenographs were obtained prior to treatment. Objective improvement consisted of a visible, measurable decrease in size of lesions, without progression of any lesion or appearance of new lesions. This regression must have existed for at least 2 months and have been observed at 2 different visits. Objective improvement was not necessarily accompanied by subjective relief. Subjective improvement was considered as any relief of pain, gain in weight, decrease of nausea and vomiting, or other relief. Several patients who were classed as subjectively improved had objective, progressive disease and succumbed to their disease or to drug toxicity even though they had stated that they were improved.

Hemograms were taken at least every other day during and for at least 2 weeks after treatment; radiographs and measurements of lesions were made usually at 2-week intervals. Patients obtaining an objective remission were retreated when possible, and an attempt was made to retreat them at 30-day intervals, though this was not always feasible. No regressions in osseous lesions were observed. In a few instances the results of treatment were so rigorous that patients refused further drug therapy, though they returned for observation. A few patients who obtained subjective relief were retreated. Retreatment was frequently better tolerated than the original course of therapy.

In most instances, the dosage schedules used were those outlined by Curreri and associates; namely, 15 mg. per kg. intravenously for 5 consecutive days followed by 7.5 mg. per kg. every other day until the development of stomatitis or leukopenia. A sore throat frequently presaged the development of stomatitis and was eventually considered as evidence for caution in treatment. The drug was well tolerated immediately in most instances, and nausea and vomiting were minimal. The drug was given orally to 4 patients at a dose of 15 mg. per kg. for longer periods than by the intravenous route.

RESULTS

5-FU was used in 60 patients with advanced malignancy and in 1 patient with Letterer-Siwe disease. All patients had been considered for conventional therapy and either had failed to respond to such therapy or were considered unsuitable. A total of 86 courses of therapy was given to 61 patients. All patients had symptoms of cancer and most were in fair condition when treated. One patient with cancer of the gall bladder and one patient with a brain tumor were moribund when treated. Twelve patients (19.6%) obtained objective remissions (table 1) by the criteria outlined, and 31 patients (50.8%) obtained some subjective relief of

symptoms. The most striking objective results were noted in patients with breast cancer, cancer of the corpus uteri, and brain tumor (table 2). Patients with objective remissions in nearly all cases were retreated and maintained improvement until resistance occurred. A few patients with subjective improvement were retreated. In the 12 patients obtaining objective benefit, some degree of toxicity resulted. In some instances, this toxicity was so severe that patients were reluctant to undergo further therapy. In most cases with remission, the benefits outweighed the toxic results. The longest period of remission, case 1, table 2, was 12 months, and this patient's breast tumor eventually failed to respond to a final toxic course of therapy. The tumor is now assumed to be resistant. Previously the patient had failed to respond to testosterone propionate given over a period of several months and refused X-ray treatment. In this and other cases with relatively long remissions, there was some regrowth of tumor between courses of therapy. One other patient (case 7, table 2) with carcinoma of the corpus uteri has had subjective improvement for a period of 10 months and diminution in size of pulmonary metastatic masses after each course of therapy. In 10 other patients the period of remission lasted from 2 to 6 months.

TABLE 1.—Summary of cases treated with 5-FU

Site of primary tumor	Number of cases	Improvement	
		Objective	Subjective
Colon	12	1	7
Breast	9	2	3
Corpus uteri	3	2	2
Tongue	2	0	1
Cervix	2	0	1
Lung	4	0	2
Stomach	4	1	3
Larynx	4	1	3
Bladder	3	1	1
Angioendothelioma*	1	0	0
Pancreas	1	0	1
Tonsil	2	0	2
Sole of foot (carcinoma)	1	1	1
Gall bladder	1	0	0
Thyroid	2	1	0
Brain tumor†	4	2	3
Myeloma	1	0	0
Reticulum-cell sarcoma‡	1	0	0
Ewing's tumor§	1	0	1
Testicle‖	1	0	0
Vulva	1	0	0
Letterer-Siwe disease¶	1	0	0
Total	61	12	31

*Locally malignant in nasopharynx.

†One patient with astrocytoma, grade II; 1 patient with glioblastoma multiforme; 2 patients with diagnosis based on pneumoencephalography and angiography.

‡Primary site in retroperitoneal area.

§Primary in lumbar spine.

‖Embryonal carcinoma.

¶Diagnosis by liver biopsy; possibly should not be considered a neoplastic disease.

TABLE 2.—Remissions after 5-FU treatment

Number	Patient	Site of primary tumor	Age	Previous treatment	Courses of treatment	Duration of remission (*months*)	Comments
1	M.M.	Breast	57	Androgen (no response)	7	12	Decrease of soft-tissue masses, healing of ulceration. Still living at 24 months. Resistant.
2	R.D.	Breast	62	X ray (response) Androgen (no response)	3	4	Decrease of large soft-tissue mass. Still living at 18 months. Resistant.
3	A.Q.	Larynx	63	X ray (no response) Surgery	2	2	Decrease in size of cervical nodes, local improvement, less dysphagia. Died in 5 months.
4	M.S.	Bladder	63	Surgery X ray (no response)	1	2+	Marked decrease in size of pelvic mass, later operated upon. Still alive at 20 months.
5	R.S.	Sole of foot—carcinoma	56	Surgery X ray (no response)	3	3.5	Marked decrease in size of inguinal nodes and amputation stump. Lived 5 months.
6	M.V.	Glioblastoma multiforme (at autopsy)	52	None	2	5	Awoke from coma, marked clearing of neurological signs, discharged home. Symptoms returned, refused further treatment. Died in 7 months.
7	M.M.	Corpus uteri	67	Surgery X ray	6	10	Definite decrease in size of pulmonary metastasis, cessation of hemoptysis. Still alive at 28 months. Resistant.
8	I.S.	Corpus uteri	56	Surgery X ray (no response)	1	2	Marked decrease in size of pelvic and abdominal masses. Refused further treatment. Died in 3 months.
9	E.C.	Thyroid	54	I^{131} Surgery X ray	1	2	Marked decrease in size of subcutaneous nodules. Refused further treatment. Died in 4 months.
10	E.H.	Stomach	60	Surgery	4	3	Marked decrease in size of abdominal masses with improvement of intestinal obstruction. Died in 4½ months.
11	M.F.	Colon	54	Surgery	1	2	Abdominal mass measureably smaller. Pain less. No further treatment given. Died in 5 months.
12	R.C.	Medulloblastoma	10	None	3	10?	Angiography and pneumoencephalography revealed "brain tumor." These signs have cleared and child is well. No proof of diagnosis. Alive at 10 months.

Toxicity

Toxic effects commonly present after intravenous therapy, but rare after oral therapy, were those noted by Curreri *et al.;* namely, stomatitis, leukopenia (agranulocytosis), anemia, nausea, vomiting, and diarrhea; the late effects were alopecia and pigmentation of the skin. In most cases, in an attempt to evaluate the drug, antibiotics and cortisone were withheld even though agranulocytosis occurred. Antibiotics were given to some patients after infection developed, and corticosteroids were added in a few other cases. Prompt application of these medications may prevent some fatalities.

Twenty-four of the 61 patients (table 3) succumbed within 30 days of treatment, giving a treatment mortality rate of 39.3 percent. An analysis of these cases revealed that 10 patients (16.4%) died as a direct result of drug therapy because of infection or delayed shock. Drug therapy may have contributed to the death of the other 14 patients, but these patients were believed to have progressive cancer which did not respond to chemotherapy. Previous intensive roentgen-ray therapy had been administered to 7 of the 10 patients who died of drug toxicity, and this therapy may have contributed to the toxicity.

Serious toxicity noted in 5 patients was delayed for 7 to 10 days after the end of treatment. Stomatitis was an indication to stop treatment, but was not invariably followed by leukopenia. Sudden agranulocytosis accompanied by shock occurred in 4 patients from 7 to 14 days after the end of treatment. These symptoms were difficult to treat or anticipate and were invariably fatal. Alopecia of some degree occurred in patients surviving more than 30 days, or who were retreated. Regrowth of hair occurred in all surviving patients.

Toxicity was much less pronounced after oral therapy, and did not occur in 4 patients receiving large doses of the drug by the oral route. Some patients developed malaise, nausea, and diarrhea, without leukopenia or stomatitis, and these symptoms were difficult to interpret. In all but 2 of the 61 patients treated, the drug was given intravenously, with resultant toxicity. Two patients with carcinoma of the colon received orally a total of 39.75 and 47.0 gm. in divided daily doses of 15 mg. per kg., without toxicity or clinical benefit. Two other patients had remission with intravenous therapy and no response to oral treatment but did develop malaise and nausea. This brief experience is at variance with that of Gold *et al.* (*5*) who found mild toxicity and some antitumor effect after oral administration.

This drug appears to have a marked toxic effect which is associated with its anticancer effect. Perhaps more experience may permit better adjustment of the dose level so that severe toxic reactions can be avoided. So far it has been impossible to predict or prevent serious toxicity and still observe a chemotherapeutic response.

TABLE 3.—Deaths within 30 days of treatment with 5-FU

Patient	Site of primary tumor	Age	Previous treatment	Survival after treatment (*Days*)	Leukocyte count prior to death	Comments
1 M.M.	Breast	42	Surgery Castration Androgen	9	3, 200	Cerebral metastasis
2 M.G.	Breast	65	Surgery X ray Androgen	4	5, 400	Cerebral metastasis
3 C.K.	Colon	54	Surgery	29	8, 400	Pulmonary insufficiency
4 S.V.	Colon	48	Surgery	22	8, 500	Pulmonary metastasis
5 J.G.	Colon	46	Surgery	22	6, 400	Intestinal obstruction and jaundice
6 J.R.	Colon	50	Surgery	15	1, 100	Pulmonary metastasis
7 P.R.	Colon	58	Surgery	25	1, 250	Intestinal obstruction
8 M.S.	Gall Bladder	63	Biopsy	3	12, 400	Jaundice and cachexia
9 J.M.	Bladder	59	Surgery X ray	6	18, 300	Hemorrhage from bladder
10 K.N.	Letterer-Siwe disease	1½	Biopsy	18	8, 500	Progressive disease
11 H.R.	Cervix	46	X ray	17	4, 600	Uremia and heart failure
12 P.R.	Lung	64	Surgery	20	5, 200	Pulmonary embolus
13 W.H.	Lung	43	Biopsy	13	1, 600	Cerebral metastasis
14 M.G.	Lung	44	Biopsy	2	7, 550	Pulmonary insufficiency
			Deaths believed related to drug therapy			
15 A.R.	Breast	46	Surgery X ray Androgen Hypophysectomy	5	850	Infection and agranulocytosis
16 R.B.	Breast	47	X ray Androgen	4	3, 400	Shock and infection
17 J.M.	Colon	69	Surgery	13	300	Leukopenia and infection
18 E.M.	Colon	47	Surgery	13	1, 000	Peritonitis and leukopenia
19 I.K.	Larynx	62	Surgery, X ray	10	850	Pneumonia and leukopenia
20 R.C.	Larynx	66	X ray	17	550	Sudden shock and leukopenia
21 L.S.	Bladder	57	Surgery X ray	14	800	Infection and leukopenia
22 H.N.	Brain	64	Surgery X ray	14	2, 500	Sudden shock. ?etiology purpura
23 J.F.	Tongue	50	X ray	15	550	Stomatitis and leukopenia
24 W.G.	Thyroid	54	Surgery Androgen	7	650	Intestinal infection, shock, and leukopenia

DISCUSSION

5-FU produced objective remissions in 9 of 22 histologic types of tumors in which it was tried. The high mortality within 30 days after the administration of the drug in part reflected drug toxicity and in part the selection of patients with advanced cancer who had previous roentgen-ray and other therapy, which were factors that undoubtedly added to the risk of this type of chemotherapy. All remissions were accompanied by some degree of drug toxicity and in some patients the toxicity was severe. In the latter part of this study, it was decided to reject seriously ill, toxic patients or those who had received intensive roentgen-ray therapy. Such a selection is essential and permits a more effective measure of the drug's usefulness especially with a drug of such high toxicity. In many cases it seemed propitious to reduce the intravenous dose to 15 mg. per kg. for 4 days rather than 5, and then to proceed with a half dose every other day until toxicity appeared. After a review of those cases in which the patient died within 30 days or less of treatment, 18 of the 24 patients would now be considered poor risks and unsuitable for treatment.

The most satisfactory remissions occurred in patients with breast and uterine tumors, and this agrees with the findings of Curreri *et al.* Remissions have lasted 12 and 10 months in 2 patients who had frequent retreatment. The low remission rate in carcinoma of the colon was disappointing in view of Curreri's results, but the number of cases may be too small for final judgment.

The treatment of 4 patients with primary brain tumors merits further comment. The first patient treated was moribund when first seen. He had previously had an operation for a glioblastoma multiforme and had received an intensive course of roentgen-ray therapy. Response was dramatic and he became well enough to walk about the wards and to feed himself. He succumbed suddenly to an unknown cause 3 weeks after the end of treatment. Postmortem examination was refused. This case could not be considered a remission, but the experience encouraged further trial of the drug in other patients. Two other patients with angiographic and clinical evidence of a brain tumor have been treated, with dramatic clinical results: (1) A female patient (case 6, table 3), who improved markedly, and tumor regression could be shown by subsequent angiographic studies. Toxicity was severe and she refused more than 2 courses of treatment; at autopsy the tumor was shown to be a glioblastoma multiforme. (2) A 10-year-old female with X-ray and clinical evidence of a brain-stem tumor believed to be a medulloblastoma. Neurological signs of tumor have nearly disappeared and pneumoencephalography shows marked improvement. She is still alive and reasonably well, but histologic proof of tumor is lacking. A fourth patient, with a histologic diagnosis of astrocytoma 9 years previously, and with a recurrence of symptoms, was treated without response. The evaluation of the chemotherapy of brain tumors presents a considerably greater problem than that of most somatic tumors, expecially when lesions are believed to be deep

in the brain or brain stem, and biopsy is hazardous. This brief experience is encouraging.

While this drug is perhaps too toxic for general use, the responses described, which were obtained after usual treatment had failed, would justify further study of related analogues. Preliminary studies already indicate that a less toxic and, perhaps, as effective preparation is available (*6*).

Four of the 9 patients with advanced breast cancer had previously experienced hypophysectomy, 3 with remission of their disease. All but one of these patients tolerated 5-FU treatment reasonably well, but none was benefited.

The remission rate obtained in this series of cases is not greatly different from that obtained with some alkylating agents providing they are used in sufficient doses. Inasmuch as 5-FU is believed to act in a different manner from X irradiation, alkylating agents, and other known effective antimetabolites in human cancer, it is conceivable that certain combinations of therapy may be more effective and less toxic. Preliminary studies with 6-mercaptopurine have been disappointing.

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Studies on Noninfective Tumors Produced in Turkeys by Rous Sarcoma Virus [1,2]

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SUMMARY

The production of noninfective tumors with low doses of Rous sarcoma virus (RSV), as originally described by Bryan, Calnan, and Moloney, in the chicken, was found to be markedly enhanced in turkey poults. Although the dilution endpoint for tumor production in both chicks and turkey poults was identical, tumors produced in turkeys with as much as 100,000 ED50 of RSV were found to be noninfective. Data are presented which show that the infecting dose of virus, age of the tumor, and virus-neutralizing factors in tumor extracts and serums were important factors influencing the infective titer of tumor tissue in turkeys. Sarcomas induced by a relatively large dose of virus yielded large and fairly uniform amounts of virus provided the tumors selected for assay were harvested within 10 to 14 days after induction. Similarly the serums of such birds were found to be free or to contain only small amounts of specific antibody. When tumors were harvested at more than 15 days after induction, they were found to contain only small amounts of virus or were noninfective. Almost invariably, a noninfective tumor induced by the larger doses of virus came from a bird whose serum contained large amounts of specific antibody. This reciprocal relationship between virus yield and antibody content of the donor bird was also dependent on the amount of initial infecting dose of RSV. That other unknown factors were also involved in the phenomenon of noninfectivity was indicated by the fact that tumors induced with less than 1 ED50 of virus were noninfective, while the serums of their hosts contained no demonstrable virus-neutralizing activity. Finally, it was found that portions of the same tumor varied greatly in the content of infective virus.—J. Nat. Cancer Inst. 25: 141–159, 1960.

NUMEROUS BIOLOGICAL studies on virus-induced tumors have revealed a marked variation in the quantity of virus that can be extracted from them. This phenomenon has been emphasized in recent reviews (*1, 2*). With Rous sarcoma, Carr (*3*) observed that slow-growing tumors induced in Brown Leghorn chickens yielded noninfective filtrates provided the

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tumors selected had been growing for more than 40 days. Subsequently, he reported (*4*) that such tumors contained an inhibitor which was apparently serum antibody. Bryan, Calnan, and Moloney (*5*) reported that the amount of infective virus obtained from chicken sarcomas was related to the infecting dose of virus. When less than 1 ED50 of virus was used, about 24 percent of such "low-dose" tumors were noninfective. Similarly, when chicks were inoculated intracerebrally with less than 1 LD50 of virus, brains removed from the relatively few chicks that died contained no infective virus, in 4 of 5 instances (*6*). Recently Prince (*7*) reported that cells derived from some noninfective chicken sarcomas failed to produce virus even after extended cultivation *in vitro*. Further, this investigator showed that the majority of clones of chick embryonic fibroblasts, previously infected with small doses of RSV, also failed in many instances to produce virus. Such clones appeared to be resistant to superinfection.

Duran-Reynals (*8*) found that tumors were produced in turkeys by RSV and that these tumors could be transferred by means of cell suspensions or extracts through 4 serial passages in young turkeys, though some loss in potency was observed. Harris (*9*) observed that though very young turkeys were susceptible to RSV they became resistant after 3 weeks of age. Previous reports from this laboratory (*10*, *11*) have shown that sarcomas produced in turkeys with as much as 100,000 ED50 of chicken-tumor virus yielded little or no infective virus, despite the fact that the dilution endpoint for tumor production was identical in chicks and turkeys. It was also shown (*10*) that serial passage of RSV in turkey poults was associated with a progressive loss in potency through the fourth passage when extracts of such tumors did not produce tumors in turkeys.

More recently it was found that virus-neutralizing factors in tumor extracts and serums, age of the tumor, and the infecting dose of virus are important factors influencing the infective titer of turkey tumor tissue (*12*). These studies have been extended and are described here in detail.

MATERIALS AND METHODS

Standard Rous sarcoma virus, prepared by differential centrifugation of chicken tumor tissue and stored at −70° C., was provided by Dr. W. Ray Bryan, of the National Cancer Institute. Unsexed Beltsville white turkey poults, 2 to 7 days of age, were supplied by a reliable local poultry breeder and were used throughout these studies. All birds were maintained at appropriate incubating temperatures and had access to food and water *ad libitum*. Immediately prior to each experiment each bird was identified by a numbered metal wing band.

Inoculation of turkeys.—Suitably diluted virus in 0.2 ml. amounts was injected subcutaneously into the left wing web with a ¾ inch, 26-gauge

hypodermic needle. The needle was inserted through the muscle into the subcutaneous tissue of the wing web, thus avoiding leakage. Each bird was examined daily for the presence of tumors at the site of inoculation, beginning on the 3d day post inoculation and continuing for 3 to 6 weeks, depending on the potency of the inoculum.

Storage and preparation of tumor extracts and serums.—Tumor-bearing turkeys were killed at various intervals after infection. Tumor tissue was removed aseptically from each bird and placed in a separate glass tube which was sealed in an oxygen flame and stored at −70° C. On the day of assay, the tissue was thawed at room temperature, weighed, and ground in a mortar with alundum. Sufficient saline, containing 2 percent inactivated normal horse serum plus 100 units of penicillin and 100 μg. of streptomycin per ml., was added to make a 10 percent homogenate by weight. The homogenate was then clarified by centrifugation at 2000 rpm for 20 minutes in a refrigerated centrifuge. For serial passages of RSV in turkeys, the crude homogenate was subjected to additional centrifugation to insure the removal of any viable cells that might have escaped disintegration during freezing, thawing, and grinding. This procedure consisted of 3 additional cycles of centrifugation carried out as follows: The 10 percent tumor-tissue homogenate was placed in a Spinco Model L ultracentrifuge and centrifuged at 9000 × g for 5 minutes. The middle third of the supernatant fluid was carefully removed after each of the successive centrifugations by means of a syringe equipped with a long needle.

In some experiments the birds were bled by cardiac puncture immediately before removal of the tumor. Serum from each bird was collected by allowing the clotted blood to remain overnight at 2° C. The recovered serums were stored at −10° C. in a mechanical freezer until the day of assay. Inactivation of either serum or tumor-tissue homogenates was accomplished by placing the material in a 56° C. water bath for 30 minutes.

Methods of assay.—An improved pock-counting method in chick embryos was used, as previously described, for the assay of infectivity (*13*). Briefly the method consisted of inoculating 0.1 ml. amounts of serial tenfold dilutions of RSV onto the chorio-allantoic membranes (CAM) of 9-day-old embryos in groups of 7 to 9. After inoculation the eggs were again placed in the upright position to return the air sac to its former location. This resulted in spreading of the inoculum over the membrane. The potency of each tumor was expressed as the log number of pock-forming units (PFU) per gram of tissue and was determined from the average number of pocks produced on the CAM of 5 to 8 eggs after inoculation of the diluted material. The virus-neutralizing capacity of a test material was expressed as a neutralization index (NI), which was determined as follows: (*a*) All serums or tumor homogenates were placed in a water bath at 56° C. for 30 minutes immediately prior to testing. (*b*) Aliquots of the serums diluted 1 : 10 were mixed with equal volumes of each of 3 serial tenfold dilutions of standard RSV. (*c*) The mixtures were allowed to stand at room temperature for 30 minutes; then 0.1 ml. of each was inoculated onto

the CAM of 6 to 9 embryonated eggs. (*d*) Control mixtures containing equal volumes of diluent and appropriate dilutions of RSV were prepared and inoculated in a manner identical with that of the test materials. (*e*) The NI was then determined by subtracting the log of the average number of PFU per ml. produced in eggs inoculated with the test material from the log of the average number of PFU per ml. produced in the control eggs. In several instances, when the diluted serum neutralized all the virus in mixtures containing the most concentrated virus, the NI was expressed as being greater than the log number of PFU contained in that mixture.

RESULTS

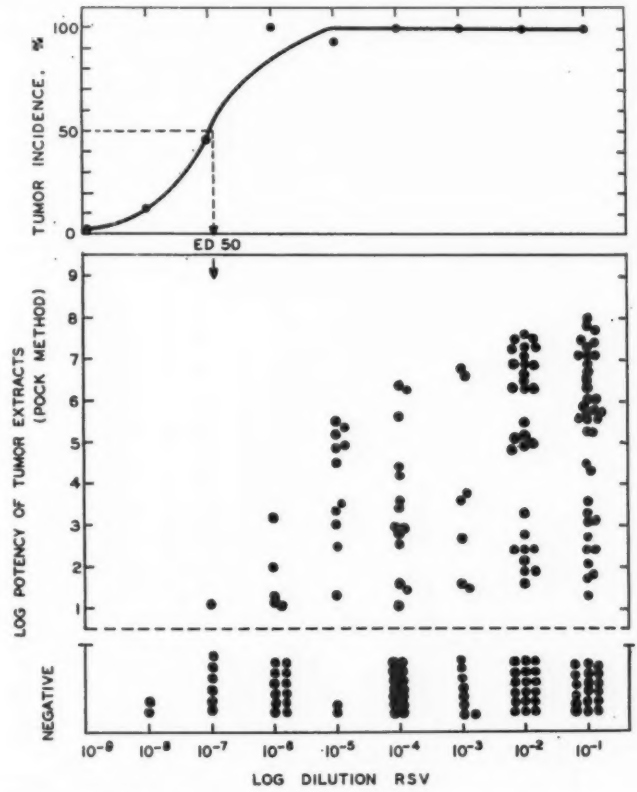
Morphological and Biological Characteristics of the Tumor

The tumors produced in turkeys at the site of inoculation of standard chicken-tumor virus differed grossly and histologically from the classical Rous sarcomas in chickens (figs. 1–8). Although the dilution endpoint for tumor production in both chicks and turkeys was identical (*10*), the sarcomas produced in turkeys appeared more rapidly than those in chickens. Once established, however, they grew considerably more slowly than those in chickens. Turkey poults infected with large doses of RSV developed readily visible, small, multiple primary tumors by the 3d day after infection. As seen in figures 1 to 4, the multiple primary tumors increased in size and eventually coalesced to form an irregular, hard, slow-growing and invasive sarcoma. When large amounts of virus were used to initiate the tumor, metastases were almost invariably found in the liver. Studies still in progress (*14*) indicate that the incidence and severity of liver metastases, time-to-death, and incidence and rate of tumor regressions following wing-web inoculation are directly related to the infecting dose of virus. Histologically sections of the tumor in turkeys showed a predominance of spindle cells and a nearly complete absence of mucoid material, which is characteristically associated with the myxosarcoma produced in chickens (figs. 5 through 8).

Relationship of Infecting Dose to Amount of Virus in Tumor Homogenates

It will be recalled that Bryan, Calnan, and Moloney (*5*) showed that the amount of infective virus demonstrable in individual chicken sarcomas was related to the amount of RSV used to initiate the tumors. The data presented in text-figure 1 show that the infecting dose of virus was also of primary importance in determining the amount of virus contained in extracts of individual turkey sarcomas. In this experiment, large groups of turkey poults were inoculated in the wing web with 0.2 ml. amounts of serial decimal dilutions of standard chicken-tumor virus. Later, turkeys were killed when their tumors were well developed and of

approximately equal size. These tumors were stored individually at −70° C. until the time of assay. The upper portion of text-figure 1 represents the incidence of tumors, in percent, in relation to the log dilution of the infecting dose of RSV. It will be seen that, as anticipated, the number of birds in which tumors developed decreased in relation to the dilution of the infecting inoculum. The ED50 of the control groups was approximately $10^{-6.8}$. The lower portion of the figure shows the infective titers of individual homogenates of tumors harvested from the respective dilution groups. Taken as a whole, it is apparent that the amount of infective virus in individual tumor extracts within a given dilution group was related to the infecting dose of virus. The points plotted beneath the dotted line refer to tumors that contained either no infective virus or an amount of virus undetectable within the limits of

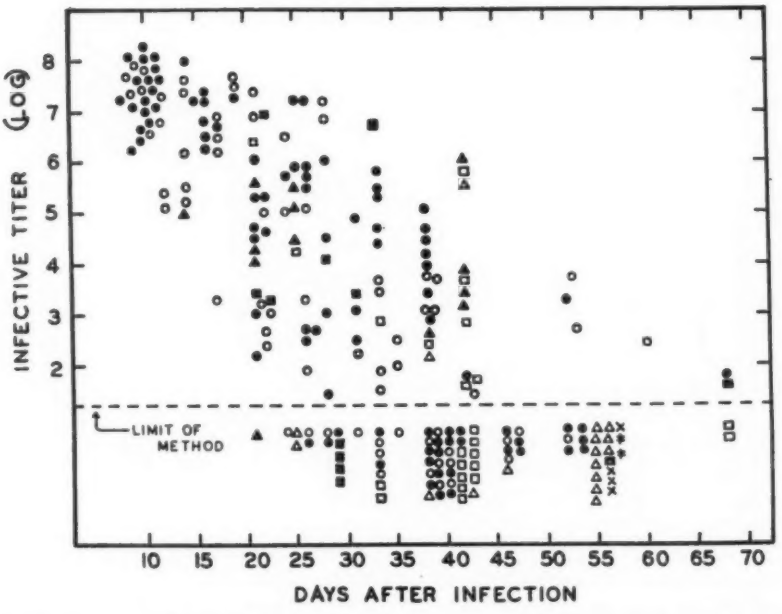


TEXT-FIGURE 1.—Effect of infecting dose of RSV on amount of infective virus demonstrable in individual turkey sarcomas. The *upper portion* shows incidence of tumors (in %) in relation to infecting dose of RSV. The *lower portion* shows results of infectivity assays of individual tumors induced with the respective doses of standard virus.

the method of assay. It is also evident that sarcomas produced with as much as 100,000 ED50 of virus yielded little or, in many cases, no demonstrable infective virus. This contrasts with results obtained in chickens and indicates that factors other than infecting dose of virus might depress the amount of infective virus in individual tumors. Further investigations were therefore carried out in search of other factors that might be involved.

Effect of Age of Tumor on Infective Virus Content

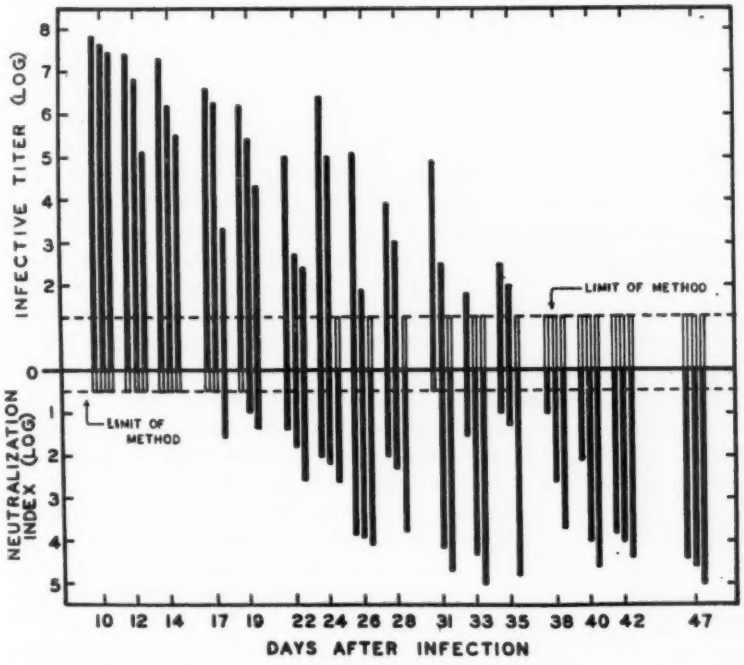
Text-figure 2 presents data which show the relationship of age of the tumor to the amount of extractable virus. The infective titers of individual tumors shown in text-figure 1 were replotted here against time, in days, between infection and removal of the tumor tissue for assay. The data show that the amount of infective virus in homogenates of individual turkey sarcomas was dependent on both the infecting dose of virus and the age of the tumor. The infective titers of tumors decreased progressively with increasing time up to approximately 35 to 40 days after infection when a large percentage of the tumors proved to be noninfective. It is also apparent that, in most cases, tumors produced with more dilute virus became noninfective more frequently than tumors produced with larger amounts of virus.



TEXT-FIGURE 2.—Effect of age of tumor on its infective virus content. The symbols refer to tumors induced with different doses of RSV, as follows: ●=10^{-1}; ○=10^{-2}; ▲=10^{-3}; △=10^{-4}; ■=10^{-5}; □=10^{-6}; ×=10^{-7}; ±=10^{-8}.

Virus-Neutralizing Factors in Serum and Tumor-Tissue Extracts

The data presented in text-figure 3 further illustrate the effect of age of the tumor on the amount of infective virus demonstrable in a given tumor homogenate and, in addition, show that the production of virus-neutralizing antibody was an important factor in this host-virus relationship. In this experiment, a large group of turkeys was infected subcutaneously with a large dose, approximately 10,000 ED50, of virus. At 10 days after infection and thereafter at the intervals indicated, both serum and tumor tissue were collected from each of 3 birds. Later, infectivity titers were determined for each tumor, and the serums of the donor birds were tested for the presence of antibody. Each bar in text-figure 3 represents quantitative assays for infectivity of the tumor tissue and for antibody in the serum of individual birds, as illustrated in the upper and lower portions of the figure, respectively. The data show that, as the infective titers of tumors decreased with time, a concomitant increase in the level of circulating antibody occurred. The serums of birds killed early were apparently free of specific antibody, but after 17 days post infection, antibody was present, though in relatively small amounts. It is also clear that in most instances a serum having a high neutralization index came



TEXT-FIGURE 3.—Effect of age of tumor on relationship of tumor infectivity to serum neutralization index of individual turkeys.

from a bird whose tumor either contained only small amounts of infective virus or was noninfective. This was especially evident in the serums and tumors of those birds killed between 38 and 47 days after infection. The tumors of such birds contained no demonstrable infective virus, whereas their serums displayed high neutralization indexes.

Additional studies (*15*), designed to determine whether the serum factor responsible for the relatively high neutralization indexes was specific antibody, revealed: (1) No loss in neutralizing activity occurred after incubation for 1 hour at 70° C., but a marked reduction followed incubation for 1 hour at 80° C. (2) The neutralizing factor was not sedimented after centrifugation at 60,000 × g for 2 hours. (3) Ammonium sulfate treatment of serum revealed the factor to be contained entirely in the globulin fraction. (4) A serum having a high NI against RSV had no apparent effect on the viruses of Newcastle disease, influenza B, fowl pox, and infectious bronchitis. (5) The time between inoculation of virus and the appearance of measurable amounts of virus-neutralizing activity and the relationship of antibody levels eventually attained to the amount of virus contained in the infecting inoculum were consistent with classical antibody responses reported for other systems.

The data presented in text-figure 3 also indicate that the level of serum antibody was not the only factor contributing to the production of noninfective tumors. This was apparent from the low serum neutralization indexes of a few of the birds whose tumors were also either free of virus or low in measurable infectivity. During these studies an observation was made which may explain this phenomenon, at least in part, namely, the infrequent occurrence of a potent, heat-labile virus-neutralizing substance demonstrable in homogenates of certain tumors. In addition to an examination of the serums and tumors for neutralizing activity and infective-virus content, respectively, aliquots of the same tumor homogenates also were assayed for specific virus-neutralizing substances. Each aliquot was rendered noninfective by heating for 30 minutes in a 56° C. water bath. Neutralization indexes were then determined for each heated homogenate in a manner identical to that used for the serums. These results are shown in table 1; the data in this table were selected to illustrate the limits of variation observed. Homogenates of tumors harvested early contained large amounts of infective virus and heated aliquots of these same homogenates displayed little or no neutralizing activity. Similarly, there was no demonstrable neutralizing activity in the serums of the donor birds. However, homogenates of tumors harvested approximately 4 weeks after infection contained little or no demonstrable infective virus, whereas they did show large amounts of neutralizing activity. With one exception, the amount of neutralizing activity in heated tumor homogenates was highly correlated with that in the homologous serum. The exception (bird #78) is an example of the occurrence, in occasional tumors, of a heat-labile inhibitor. A third aliquot of this tumor homogenate was rendered noninfective by sedimenting the virus during high-speed centrifugation

(105,000 × g). A neutralization index was then determined for the unheated, noninfective supernatant fluid and was found to be 4.21, *i.e.*, almost 4 logs more potent than a heated aliquot of the same tumor homogenate. This example is considered to be significant inasmuch as supporting data from other experiments demonstrated the inhibitor in approximately 2 percent of those tumors tested. The activity was lost after incubation of such homogenates at 56° C. for 30 minutes and, apparently, was present only in tumors which were induced with large amounts of virus but which on extraction and assay were found to yield barely detectable amounts of virus. Although the exact nature of this virus-neutralizing factor remains obscure, an example of it has been given to indicate that factors, other than those described, may be concerned with the production of noninfective tumors in the turkey.

TABLE 1.—Selected examples of the relation between infective titers of tumors and virus-neutralizing factors in heated tumor extracts and serums

Bird No.	Time after infection (*days*)	Size of tumor*	Infective titer (*log*)	Neutralizing index of:†	
				Tumor extract (*log*)	Serum (*log*)
30	10	+	7. 6	0	0
97	14	++	5. 5	0	0
39	17	++	6. 3	0	0
111	22	+++	5. 0	2. 03	2. 54
110	31	+++	4. 9	0	0
33	33	+++	1. 6	1. 37	1. 20
69	35	++	<1. 3	1. 10	1. 16
78	35	+++	1. 9	0. 42	1. 43
87	38	+++	<1. 3	0. 88	0. 91
116	40	++++	<1. 3	>3. 91	>3. 91
124	47	++++	<1. 3	>4. 32	>4. 32
104	47	++	<1. 3	>4. 32	>4. 32

*Size: + = 6 to 8 mm. in diameter, confined to wing web; ++ = 9 to 15 mm. in diameter, with frequent invasion of adjacent muscle; +++ = 16 to 30 mm. in diameter with extensive invasion of surrounding tissue; ++++ = > 30 mm.

†Tumor extracts and serums heated at 56° C. for 30 minutes.

Variation of Virus Content Within Tumors

The data presented in table 2 show that not only does viral content vary from one tumor to another but also different portions of the same tumor may vary tremendously. These data were compiled from tumor-tissue assays carried out at different times, with the same preparation of RSV as the reference standard. They were selected to illustrate the relationship of variation within tumors to tumor age. Two to 3-day-old turkey poults were inoculated in the wing web with large doses of RSV, by the usual method. Birds were killed at various intervals after infection and their tumors were removed and processed as follows: (*a*) Each tumor was divided into 2 to 4 portions, depending on its size. (*b*) A separate, clean, sterile scalpel was used to make each cut, in an attempt to prevent transferring virus from one portion to another. (*c*) Each

portion was placed in a separate glass tube, sealed, and stored at −70° C. until the time of assay. The data (table 2) show that homogenates of portions of tumors harvested early (tumors A and B) contained large and relatively uniform amounts of virus. However, portions of tumors harvested at later times showed marked differences in viral content. This was especially apparent in tumor E, in which there was a difference of more than 5 logs of virus among portions of the same tumor.

TABLE 2.—Variation of viral content within different portions of same tumor

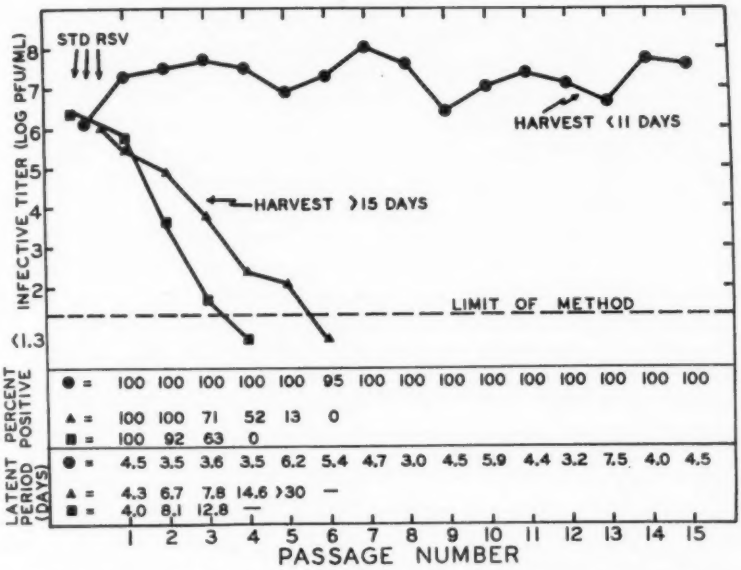
Tumor	Time after infection (*days*)	Size of tumor*	Infective titer of portion (*log*): †			
			1	2	3	4
A	10	++	7.2	7.8	—	—
B	18	+++	7.3	7.0	7.0	—
C	24	+++	6.3	3.9	—	—
D	24	+++	6.1	3.7	7.0	5.6
E	31	+++	5.5	6.3	2.1	$\leq$1.3
F	38	++++	1.6	$\leq$1.3	$\leq$1.3	$\leq$1.3
G	42	++++	1.7	$\leq$1.3	$\leq$1.3	$\leq$1.3
H	42	++++	2.8	$\leq$1.3	$\leq$1.3	$\leq$1.3
I	42	++++	$\leq$1.3	$\leq$1.3	$\leq$1.3	$\leq$1.3

*Size: + = 6 to 8 mm. in diameter, confined to wing web; ++ = 9 to 15 mm. in diameter, with frequent invasion of adjacent muscle; +++ = 16 to 30 mm. in diameter, with extensive invasion of surrounding tissues; ++++ = >30 mm.

† Log number of pock-forming units per gram of tissue.

Serial Passage of RSV in Turkey Poults

Previously it was reported from this laboratory (*10*) that serial passage of RSV in turkeys was associated with progressive loss in potency with each passage until the fourth passage, when the tumor extracts were noninfective. The studies described suggested that both the development of neutralizing antibody and the effect of infecting dose on the yield of extractable virus contributed to this failure to pass the virus serially. Accordingly, a second attempt at serial passage was made by using for each passage tumors harvested within the first 10 days after induction, presumably before the appearance of virus-neutralizing antibody. Conversely two separate control serial passages were made in which each tumor selected for serial passage was from a bird which had been inoculated 16 to 30 days previously. The results of these experiments are presented in text-figure 4. Standard RSV derived from chicken tumor tissue was used to initiate each passage in turkeys. For the subsequent passages, 10 percent tissue extracts were prepared from pools of 3 to 4 turkey tumors. The methods employed for preparing the inoculums are described under "Materials and Methods." The data show that, as predicted from the relationships revealed in text-figure 2, serial passage of standard RSV was readily accomplished provided the tumors selected for passage were harvested early. It is also clear that in both series of passages that utilized tumors harvested more than 15 days after induction, the progressive decrease in extractable virus per passage was concomitant with an increase in the latent period for tumor production.



TEXT-FIGURE 4.—Serial passage of RSV in the wing webs of turkey poults.

DISCUSSION

The fact that noninfective tumors may be produced in chickens or turkeys by an agent whose oncogenic properties are well established is of obvious importance in attempts to isolate similar agents from neoplasms of unknown etiology. The data presented in this paper show that noninfective virus-induced tumors can be produced in turkeys in a predictable manner. It is clear that the infecting dose of virus and the age of the tumor profoundly influence the amount of infective virus in extracts of experimental tumors. It is also apparent, however, that the age of the tumor was not a virus-depressing factor *per se*, but rather, when tumors were harvested at a later time, potent virus-neutralizing substances in tumor extracts and serums were produced in amounts that increased with the number of days elapsing between infection and removal of the tumor tissue for assay. The production in turkeys of levels of circulating antibody capable of neutralizing 5 to 6 logs of added active RSV is contrary to numerous observations on the antigenicity of this virus in chickens and would appear to explain, at least in part, the higher frequency of noninfective tumors in turkeys. One explanation for the difference in antibody levels between the two species may lie in the fact that the turkey tumors grew much more slowly and that the tumor-bearing birds therefore survived substantially longer than chicks subjected to the same experimental treatment. It is probable that the slower growth of the tumors in turkeys provides a more prolonged antigenic stimulus

which results in the production and maintenance of higher levels of circulating antibody.

Although the production of large amounts of serum antibody appeared to be the most important factor contributing to the lack of infectivity of tumors in turkeys, the observation that noninfective tumors produced in turkeys with less than 1 ED50 of virus (*11*) contained no neutralizing substances suggested that other unknown factors were also involved. Heat-labile inhibitors occasionally found in extracts of tumors initiated with relatively large doses of virus apparently do not exist in those induced with such small amounts of virus. A mechanism possibly associated with the failure to recover virus from low-dose tumors may involve the production of incomplete, noninfective virus in such tumors. The latter, conceivably, could function as an interfering agent during the infectivity assay. Although this possibility is at present only hypothetical, it has been reported that heat- or ultraviolet-inactivated RSV markedly lowered the cytopathogenic effect of active virus in tissue cultures (*16*) and the formation of pocks in chick embryos (*17*).

The marked variation in the amount of infective virus contained in portions of the same tumor suggests a situation in which more than one contributing factor is operative. Studies still in progress have shown that, in addition to the differences in total infective-virus content, different portions of the same tumor also may show significant variations in content of neutralizing substances. In most cases, low levels of extractable virus were associated with relatively high levels of neutralizing activity. Occasionally, however, noninfective homogenates showed no measurable virus-neutralizing activity.

The important question of whether or not noninfective tumors produced in turkeys are actually virus-free cannot be answered with certainty at the present. Reports from other laboratories appear to shed some light on this question. Calnan *et al.* (*18*), in attempting to establish a low-dose tumor line by serially transplanting noninfective Rous sarcoma tissue in chickens, found that after a number of serial transplants the tumors reverted to the characteristic rapidly growing and highly invasive type from which large amounts of virus were easily extracted. Similarly, Prince (*7*) has reported that following cultivation *in vitro* of trypsinized cells from noninfective chicken tumors, infective virus was released into the culture fluids in some instances. Unlike the noninfective chicken sarcomas which were produced only with low doses of virus, tumors produced in turkeys with as much as 100,000 ED50 of RSV frequently yielded no virus on extraction. The primary reason for this difference is clearly the presence, in turkeys, of large amounts of circulating antibody which combines with virus liberated from cells during the process of mechanical grinding and extraction. In this regard, it is significant that serial passage of RSV in the wing web of turkeys was accomplished only when the tumor tissue of each preceding passage had been harvested within 10 days after infection, that is, before the appearance of detectable amounts of serum antibody.

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PLATE 22

FIGURE 1.—Sarcoma in wing web of 11-day-old turkey poult, 5 days after inoculation of approximately 10,000 ED50 of Rous sarcoma virus (standard RSV, lot CT 694). *Note* tumor arises as small, multiple nodules in wing web. Nodules eventually coalesce, at which time tumor invades adjacent muscle and increases in size (figs. 2 to 4).

FIGURE 2.—Same as figure 1; 11 days after infection.

METRIC SYSTEM
2
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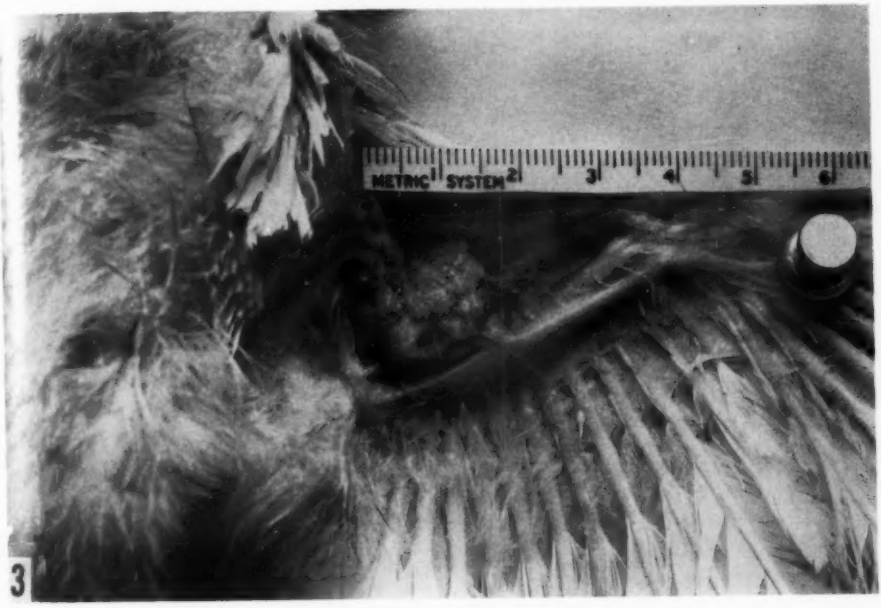
PLATE 23

FIGURE 3.—Same as figure 1; 18 days after infection.

FIGURE 4.—Same as figure 1; 31 days after infection.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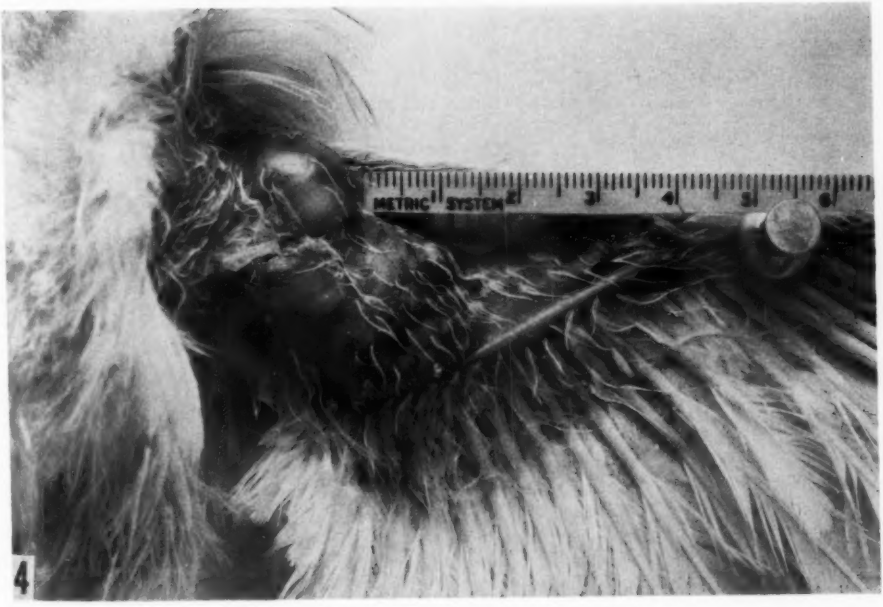
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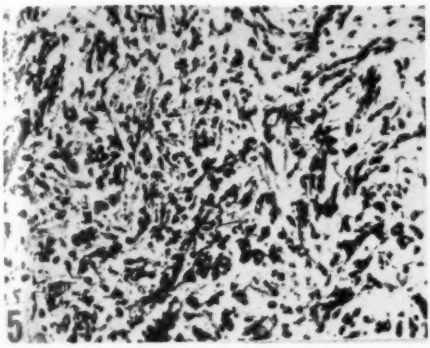
PLATE 24

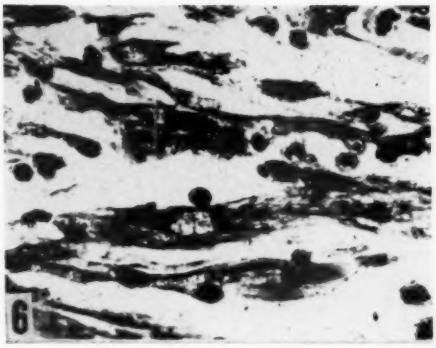
FIGURE 5.—Section of 14-day-old chick tumor produced by wing-web inoculation of approximately 10,000 ED50 of Rous sarcoma virus (standard RSV, lot CT 694). *Note* relatively loose structure of this classical myxosarcoma, which is composed of both round- and spindle-type cells. × 100

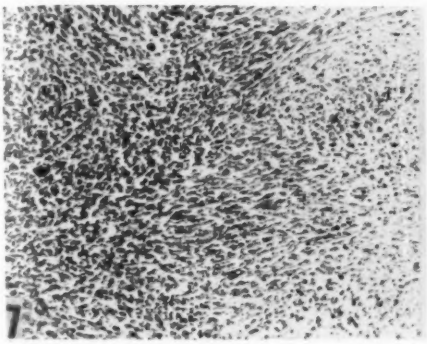
FIGURE 6.—Same as figure 5. × 450

FIGURE 7.—Section of 14-day-old turkey tumor produced by wing-web inoculation of approximately 10,000 ED50 of Rous sarcoma virus (standard RSV, lot CT 694). *Note* more dense structure of this tumor and predominance of spindle cells. × 100

FIGURE 8.—Same as figure 7. × 450







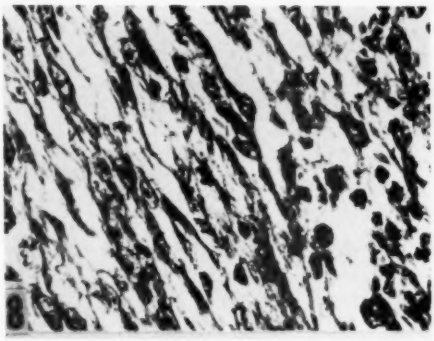


PLATE 24

FIGURE 5.—Section of 14-day-old chick tumor produced by wing-web inoculation of approximately 10,000 ED50 of Rous sarcoma virus (standard RSV, lot CT 694). *Note* relatively loose structure of this classical myxosarcoma, which is composed of both round- and spindle-type cells. × 100

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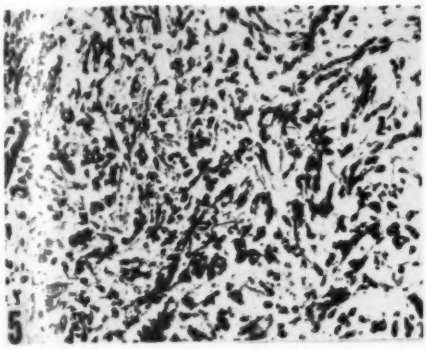
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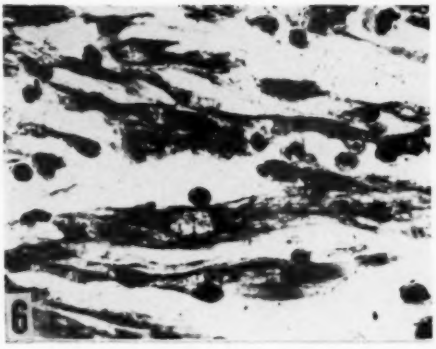
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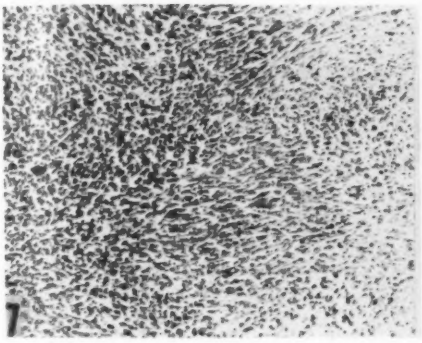
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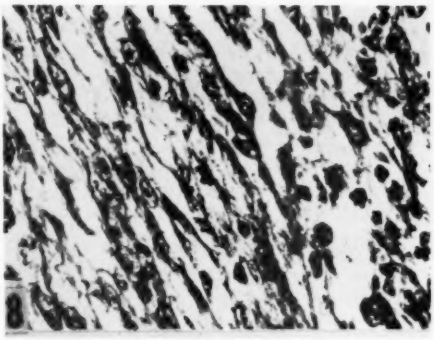
8

Influence of CO_2 Tension on the Respiration of Yoshida Ascites Tumor Cells [1,2]

JØRGEN KIELER,[3,4] *Fibiger Laboratory, Biological Division, Copenhagen, Denmark*

SUMMARY

The mechanism of the stimulating effect of CO_2 on the respiration of Yoshida ascites tumor cells was studied by a modified Cartesian diver technique. The effect of CO_2 on the endogenous respiration of Yoshida cells was most pronounced in tumors with a high mitotic coefficient. In the presence of glucose the relative effect was higher than in its absence. This could be explained in part by a neutralization of the Crabtree effect. Pyruvate added to the medium increased the effect, while oxalacetate made the cells independent of CO_2. These results suggest that the stimulatory effect of CO_2 on the respiration of Yoshida cells is due to an increased rate of oxalacetate synthesis.—J. Nat. Cancer Inst. 25: 161–176, 1960.

FOR A number of years, it has been realized that carbon dioxide is not an entirely inert end product of animal-cell metabolism. Biochemical investigations (*1*) have revealed a number of primary CO_2 fixation reactions, the physiological significance of which, however, has not yet been established.

The influence of carbon dioxide/bicarbonate on cell growth in tissue culture has been studied by various authors. Harris (*2*) and Geyer and Chang (*3*) found a direct growth-supporting effect of CO_2/HCO_3^-, while Eagle (*4*) failed to demonstrate any bicarbonate requirement for cell growth *in vitro*. A growth-inhibiting effect of CO_2 *in vivo* has recently been postulated by Loomis (*5*).

Several investigators have found that various animal cells show a higher rate of respiration in a CO_2/HCO_3^- buffer than in a phosphate buffer. These findings have previously been reviewed (*6*, *7*). In this laboratory, studies on Yoshida ascites tumor cells (*6*, *8*), Earle's strain L mouse fibroblasts (*6*), and normal and malignant human leukocytes (*7*) have shown maximal respiration in Ringer-Locke's solution when

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[2] This study was supported by grants from the Anders Hasselbalch Anti-Leukemia Fund, the Danish National Research Foundation, King Christian X's Memorial Fund, and the Leukemia Society, Inc.

[3] With the technical assistance of Kirsten Stenbøg.

[4] The author wants to thank "Løvens Kemisk Fabrik" for supplying the rats in this investigation.

the latter is brought into equilibrium with a gas phase containing 0.5 to 2 percent CO_2. These findings are summarized in table 1.

In the present report, previously unpublished technical details are described together with the results of some experiments carried out to elucidate the mechanism of the stimulatory effect of CO_2 on the respiration of Yoshida ascites tumor cells.

MATERIALS AND METHODS

The Yoshida ascites tumor was carried in noninbred albino rats of the Leo stock. In most experiments the animals were killed by cervical dislocation, 3 to 5 days after transplantation, and the ascitic fluid was aspirated under sterile conditions. Only nonhemorrhagic material was used. The cells were suspended directly in the experimental media without previous centrifugation and washing, which was found to have a damaging influence on cell respiration. By dilution, the cell concentration was adjusted to 4 to 6 thousand cells per μl. The contaminant ascitic fluid never amounted to more than one tenth of the final solution.

Ringer-Locke's solution, with various substrates, buffered with tris(hydroxymethyl)aminomethane at a concentration of 30 mM served as experimental medium. Small amounts of penicillin (500 I.U./ml.) and streptomycin (0.05 mg./ml.) were added. The concentration of $NaHCO_3$ was made up to correspond to the CO_2 concentration of the gas phase at *p*H 7.4, and the NaCl concentration was varied so as to maintain constant osmotic pressure.

Cellular respiration was measured with the Cartesian diver technique developed by Linderstrøm-Lang (*9*) and Holter (*10*). To maintain a constant CO_2 tension in the diver during the experiment, the usual CO_2-absorbing NaOH seal was replaced by a solution of sodium bicarbonate. Theoretically it would have been desirable to make the bicarbonate solution as concentrated as possible, but due to the risk of distillation, the concentration could not be increased above 0.15 M.

The Ringer-Locke's solutions and the bicarbonate solutions were gassed for 4 to 6 hours at 37° C., with the gas phase to be studied, and stored in ampoules after control of *p*H. Before the experiments, the Ringer-Locke's solution and the bicarbonate solution were poured into 2 open vessels placed in a larger vessel, which was tightly closed with a stopper provided with an inlet and an outlet. Renewed gassing was then carried out for 10 to 15 minutes, after which the inlet and the outlet were closed. Corking of the larger vessel after gassing was avoided in order not to create an overpressure, which was found to make the ensuing establishment of equilibrium in the divers difficult. The solutions were left for equilibration overnight at 37° C.

The filling of the divers was carried out at 37° C. A schematic drawing of the filling equipment[6] is shown in text-figure 1. The diver (J) is fixed

[6] Made by Ole Dich, Brøndby Strand, Denmark.

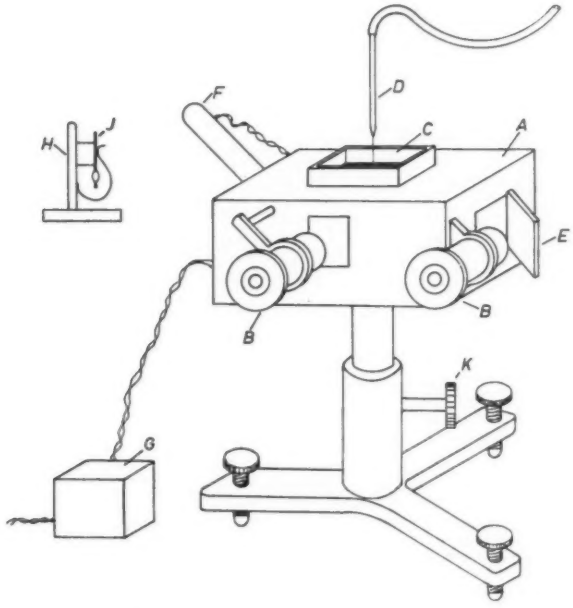
TABLE 1.—Influence of CO_2 on cellular respiration

CO_2%:	O_2 consumption per cell per hour ($\mu l. \times 10^{-6}$) 0	0.03	0.50	1.00	2.00	2.50	3.00	4.00	5.00	Reference
Yoshida tumor*		2.33	2.75	3.62	3.42		2.42		2.80	(6)
Strain L*	2.77	4.29	5.49	5.35	5.64	5.53	5.61	4.83	5.27	(6)
Human leukocytes†(*a*)		0.26	0.31	0.32	0.32		0.28		0.23	(7)
—normal†(*b*)		0.19	0.26	0.27	0.26		0.22		0.18	(7)
Human leukocytes†(*a*)		0.22	0.28	0.27	0.26		0.24		0.20	(7)
—myeloid leukemia†(*b*)		0.10	0.17	0.17	0.16		0.13		0.11	(7)
Human leukocytes†(*a*)		0.23	0.25	0.30	0.28		0.26		0.20	(7)
—lymphocytic leukemia†(*b*)		0.11	0.15	0.18	0.16		0.13		0.11	(7)

*Medium: Ringer-Locke's solution with 5 mM glucose.

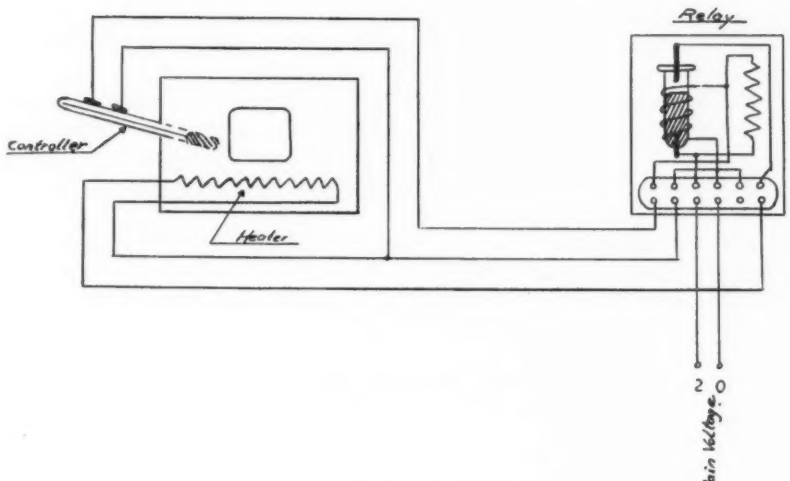
†Medium: Ringer-Locke's solution without glucose: (*a*) average respiration during the first 3 hours of incubation; (*b*) average respiration during the 4th to 6th hour of incubation.

in a clamp (H) which is placed under water in a cuvette (C) fitting into an aluminum thermostat (A). The diver in the cuvette can be watched from 2 sides, by 2 magnifying lenses (B), through a hole in the front wall and another hole in the side wall of the thermostat. The hole in the side wall has a mirror (E). The heater, which is connected with a relay (G), is placed in the bottom of the thermostat, and the thermocontroller (F) at the other side wall. A diagram of the heating system is shown in text-figure 2. The thermostat is mounted on a stand which may be raised and lowered by means of a rack and pinion (K). The tripod base of the stand rests on adjustment screws so that its inclination may be changed. The filling of the diver is accomplished by fixed pipettes (D), as described by Holter (*10*), whose original publication should be consulted for further technical details.



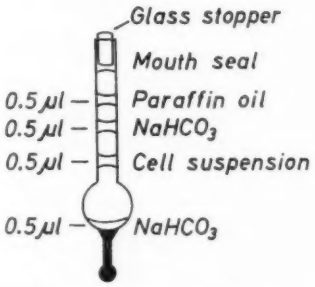
TEXT-FIGURE 1.—Filling thermostat.

The first solution to be introduced into the diver was 0.5 μl. of bicarbonate which was placed at the bottom of the diver (*see* text-fig. 3). The interior of the diver was then gassed for 2 to 3 minutes, with the gas phase to be studied. This procedure was repeated after the introduction of each of the subsequent neck seals, the tip of the gassing pipette being placed just above the surface of the seal, which was introduced last. As the first neck seal, 0.5 μl. of the cell suspension was placed at the lower end of the neck. About 1 mm. above the cell suspension, another 0.5 μl. of bicarbonate solution was placed as a second neck seal. A third neck



TEXT-FIGURE 2.—Diagram of heating system of filling thermostat.

seal was made up of 0.5 µl. of paraffin oil, and when all these seals had been introduced, the mouth of the diver was temporarily closed by a very small water seal.



TEXT-FIGURE 3.—The charged diver.

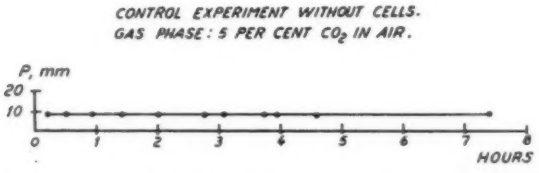
The diver was then transferred to the flotation vessel, where it would float just beneath the surface of the flotation medium. With a fine pipette, the tip of which was placed between the oil seal and the provisionary water seal, enough gas was sucked out to make the diver sink slowly to the bottom. After rinsing of the mouth seal to replace the water with flotation medium, the mouth seal was provided with a hollow glass stopper, which has been shown by Linderstrøm-Lang and Holter (*11*) to be an effective means of reducing gas diffusion. All other technical details have been described by Holter (*10*).

The divers used had a total volume of about 10 µl. Each of them usually contained 2 to 3 thousand cells, which in most cases showed a

linear respiration curve during the first 2 to 4 hours of incubation. Later, the curves fell off gradually. In general, the experiments were run for 5 to 8 hours.

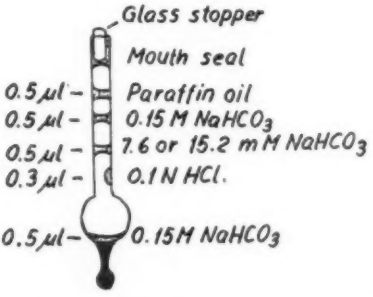
The maintenance of a constant CO_2 tension in the gas phase is mainly dependent on two factors: (1) No leakage of CO_2 from the diver should occur during the experimental period, and (2) the CO_2-absorbing capacity of the bicarbonate solution should be sufficient to prevent any accumulation of metabolic CO_2 in the diver.

To make sure that no loss of CO_2 would occur from the divers during the experimental period, all divers with their stoppers were tested in control experiments with 5 percent CO_2. Text-figure 4 shows that no measurable loss of CO_2 occurred.



TEXT-FIGURE 4.—Control experiment for testing the CO_2 leakage.

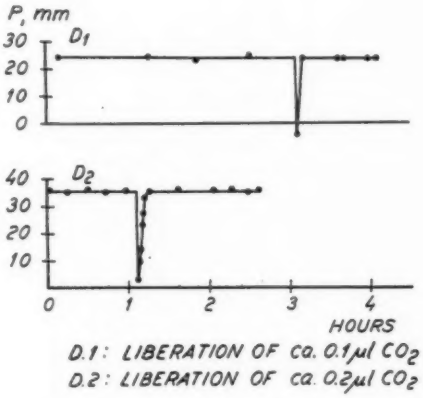
To test the CO_2-absorbing capacity of the bicarbonate solution, the following experiment was carried out: Divers were charged with 2 × 0.5 µl. of 0.15 M $NaHCO_3$ solution, as described, in addition to the usual oil seal and mouth seal provided with a stopper. Instead of the cell suspension, 0.5 µl. of a 7.6 or a 15.2 mM $NaHCO_3$ solution was inserted as a seal at the lower end of the neck. Just below this seal, 0.3 µl. of 0.1 N HCl was placed as a side drop on the wall of the neck as shown in text-figure 5. The initial gas phase was room air.



TEXT-FIGURE 5.—Diver charged with $NaHCO_3$ and HCl for testing the CO_2-absorbing capacity.

When the divers had been charged, they were transferred to the flotation vessels, and the equilibrium pressure was measured several times over a period of at least 1 hour. The HCl side drop and the 7.6 or 15.2 mM

$NaHCO_3$ seal were then made to coalesce by a suitable increase of the pressure. The effect of the ensuing liberation of CO_2 was studied by manometric measurements at very short intervals. From text-figure 6 it appears that an accumulation of CO_2 occurred, indicated by a change of the equilibrium pressure. After 6 to 10 minutes, however, the equilibrium had returned to the original level. This means that in 6 to 10 minutes about 0.1 to 0.2 µl. of CO_2 can be absorbed by the $NaHCO_3$ seals, which is about 100 times more than the amount of CO_2 produced in the same time by 3,000 Yoshida ascites tumor cells showing maximal respiration. It also shows that even if all the bicarbonate of the experimental medium with the highest bicarbonate concentration studied (15.2 mM) was liberated as CO_2 due to *e.g.*, glycolysis, the CO_2 accumulation *per se* can at most give rise to a deviation of the respiration curve of 10 minutes' duration.



TEXT-FIGURE 6.—CO_2-absorbing capacity of 1 µl. 0.15 M $NaHCO_3$ solution in the Cartesian diver.

RESULTS

To elucidate the mechanism of the stimulatory effect of CO_2 on the respiration of the Yoshida cells, an investigation of the relationship between this effect and various substrates was planned. As a basis for this investigation, the effect of CO_2 on the endogenous respiration of Yoshida ascites tumor cells suspended in Ringer-Locke's solution without any substrate was first studied. During these experiments it was found that the results obtained were greatly influenced by the age of the tumor. In table 2 the results of 1 of 3 experiments are shown, which demonstrate this relationship between tumor age and CO_2 effect.

The cells were obtained from a rat, which was killed 6 days after transplantation. The endogenous respiration was studied at room air and at 1 percent CO_2, and a mitotic count was made on a smear preparation,

TABLE 2.—Changes in the response of Yoshida ascites tumor cells to CO_2 during their growth *in vivo*

	Oxygen consumption per cell per hour ($\mu l. \times 10^{-6}$)											
Days after transplantations:	0			2			4			7		
Composition of gas phase:	Abs.	Avg.	Rel.* (*percent*)	Abs.	Avg.	Rel.* (*percent*)	Abs.	Avg.	Rel.* (*percent*)	Abs.	Avg.	Rel.* (*percent*)
Room air (0.03% CO_2)	2. 95	2. 95	100	1. 28 1. 18	1. 23	100	2. 40 2. 36	2. 38	100	3. 53	3. 53	100
1% CO_2 + 20% O_2 + 79% N_2	4. 22 3. 97	4. 10	139	2. 70 2. 76	2. 73	222	5. 34 5. 36	5. 35	225	4. 74 4. 75	4. 75	135
Mitotic coefficient†		6			10			19			3	

* Percent of respiration at room air.
† Number of mitoses per 1,000 nondividing cells.

the number of mitotic figures being counted in 1,000 cells. It appears from table 2 that 1 percent CO_2 stimulated the endogenous respiration of these cells by 39 percent. The mitotic coefficient was 6. The cells were transplanted intraperitoneally to a new rat from which 0.2 to 0.3 ml. ascitic fluid was aspirated 2, 4, and 7 days after transplantation. Each time a study was made of the mitotic coefficient, and of the endogenous respiration at room air and at 1 percent CO_2. As shown in the table, duplicate determinations were carried out in most cases.

A considerable decrease in the respiration at room air was found on the 2d day; but the relative effect of CO_2 went up to 122 percent and the mitotic coefficient to 10. On the 4th day, the endogenous respiration at room air rose again, while the relative effect of CO_2 remained unchanged. The mitotic coefficient had gone up to 19. On the 7th day a further increase in the respiration was seen, but the relative effect of CO_2 as well as the mitotic coefficient had gone down. Thus a pronounced effect of CO_2 coincided with a high mitotic activity.

On the basis of these findings, it seemed preferable to use cells aspirated 2 to 4 days after transplantation for the study of the CO_2/substrate relationship. However in spite of all attempts to standardize the transplantation procedures, a uniform rate of tumor growth usually could not be obtained. This may be explained in part by the fact that the ascites tumor was carried in noninbred animals. Consequently great variations in the rate of respiration and in the effect of CO_2 were seen, even when the cells were taken exclusively on the 4th day after transplantation.

However if the cells were selected according to their rate of respiration at room air, uniform and reproducible results could easily be obtained. Table 3 shows the effect of CO_2 on the endogenous respiration of 3 groups of cells: (1) those with an oxygen consumption of less than 2×10^{-6} μl. per cell per hour at room air; (2) those with an oxygen consumption of 2 to 3×10^{-6} μl.; and (3) those with an oxygen consumption of more than 3×10^{-6} μl. per cell per hour at room air. CO_2 had a stimulatory effect in the first 2 groups, which according to Student's t test was statistically significant (P: group I = 97.5–99%; group II = 95–97.5%). In the last group with an oxygen consumption of more than 3×10^{-6} μl. at room air, CO_2 had on an average a slight but statistically insignificant inhibitory effect (P = 90–95%). Therefore cells belonging to the first 2 groups were preferred in the following experiments.

The effect of 1 percent CO_2 on the respiration of the Yoshida cells suspended in Ringer-Locke's solution with glucose at a concentration of 5 mM is shown in table 4. In both groups, CO_2 had a significant stimulatory effect (P: group I = 99.95%; group II = 99.5–99.9%).

For each of the individual experiments, the average results of which are shown in tables 3 and 4, the percentage increase in respiration due to CO_2 was calculated. From table 5, it appears that the average percentage stimulation by CO_2 in group I was significantly higher in the presence of glucose than in its absence (P = 99.95%), while there was no difference in group II.

TABLE 3.—Effect of CO_2 on the endogenous respiration of Yoshida ascites tumor cells

Expt. group*	Number of experiments	Maximal O_2 consumption ($\mu l. \times 10^{-6}$)/cell/hour			
		Air	1 Percent CO_2	t	f
I	5	1. 37	1. 87	2. 454	8
II	7	2. 41	3. 43	2. 146	12
III	10	3. 44	3. 11	1. 522	18

*I: Respiration in air less than 2×10^{-6} µl. per cell per hour; II: respiration in air $2\text{-}3 \times 10^{-6}$ µl. per cell per hour; III: respiration in air more than 3×10^{-6} µl. per cell per hour.

TABLE 4.—Effect of CO_2 on respiration of Yoshida ascites tumor cells in Ringer-Locke's solution with 5 mM glucose

Expt. group*	Number of experiments	Avg. max. O_2 consumption/cell/hour ($\mu l. \times 10^{-6}$)			
		Air	1 Percent CO_2	t	f
I	6	1. 53	2. 84	4. 673	10
II	6	2. 39	3. 26	3. 336	10

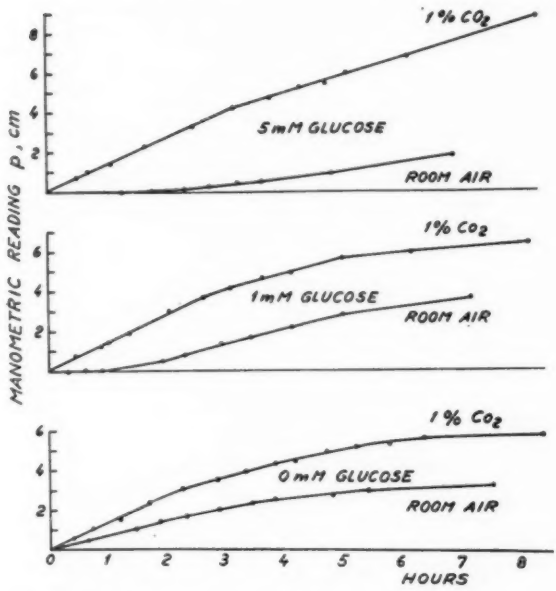
*I and II: *see* table 3 (measured in the presence of glucose).

TABLE 5.—Relative effect of CO_2 on respiration of Yoshida ascites tumor cells at 0 and 5 mM glucose

Expt. group*	Percent stimulation of 1 percent CO_2 compared with respiration in air			
	0 mM glucose	5 mM glucose	t	f
I	40	88	8. 4068	9
II	43	38	0. 2148	11

*I and II: *see* table 3.

A study of the respiration curves shows that the greater relative effect of CO_2 in the presence of glucose may be explained in part by a neutralization of the Crabtree effect. Text-figure 7 shows the respiration curves obtained at room air and at 1 percent CO_2 with Yoshida cells suspended in Ringer-Locke's solution, with glucose at concentrations of 0, 1, and 5 mM. The curves have been corrected so as to represent divers of identical size (V=7.0) and with an equal number of cells (2,000). Furthermore by extrapolation back from the first manometric readings to the time when the cells were suspended in the experimental media, the curves have been made to start at zero time. In the absence of glucose there is no Crabtree effect but a moderate stimulatory effect of CO_2. At 1 mM glucose, the Crabtree effect shows itself at room air as a complete inhibition of the respiration during the 1st hour. This inhibition gradually disappears during the 2d hour of incubation. However at 1 percent CO_2 there is no visible Crabtree effect. This difference is even more pronounced at 5 mM glucose when the Crabtree effect lasts for at least 4 hours at room air, but when there is no sign of any Crabtree effect, at 1 percent CO_2. This neutralizing effect of CO_2 on the Crabtree effect was found repeatedly, though it was not a complete neutralization in all experiments.

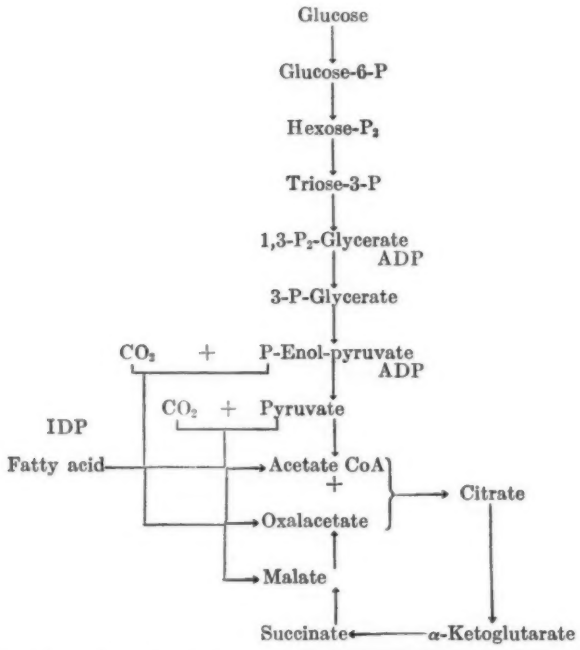


TEXT-FIGURE 7.—Effect of CO_2 on the respiration of Yoshida cells at different glucose concentrations.

Thus the greater relative effect of CO_2 in the presence of glucose than in its absence is explained in part by the influence of CO_2 on the Crabtree effect. This finding suggests an adenosine diphosphate sparing effect of CO_2 which could be explained by a reaction between phospho-enol-pyruvate and CO_2 through which oxalacetate is formed (*12*), as shown in text-figure 8.

An increased formation of oxalacetate may also explain the effect of CO_2 on the endogenous respiration of the Yoshida cells. Since phospho-enol-pyruvate is not readily absorbed by the cells, the relationship between this substrate and the CO_2 effect could not be studied in intact cells. CO_2, however, may also react with pyruvate to form oxalacetate via malate (*13*), and a relationship between this substrate and the CO_2 effect would therefore indicate oxalacetate as the final active factor responsible for the stimulatory effect of CO_2 on cellular respiration.

From table 6 it appears that an increase in the stimulatory effect of CO_2 was obtained when pyruvate was added to the medium at a concentration of 5 mM. In some experiments this relationship could only be demonstrated after 2 to 3 hours of preincubation in Ringer-Locke's solution without any substrate, indicating the presence of an endogenous source of pyruvate which should first be exhausted before the effect of added pyruvate became evident. In similar experiments with acetate added to the medium after 2 hours of preincubation no relationship between the stimulatory effect of CO_2 and added substrate could be



TEXT-FIGURE 8.—Some steps in the intermediary carbohydrate metabolism.

TABLE 6.—Effect of pyruvate and CO_2 on the respiration of Yoshida cells

Pyruvate concentration:			0 mM		5 mM		
Expt. group	Expt. No.*	Gas phase	Respiration†	Percent stimulation	Respiration†	Percent stimulation	Percent stimulation by pyruvate
I	1	Air	0.88		1.13		28
		1% CO_2	1.56	77	2.28	102	46
I	2	Air	0.60		0.52		−14
		1% CO_2	0.65	8	0.98	88	51
II	3	Air	2.79		3.13		12
		1% CO_2	2.86	3	3.82	22	33

*Expt. 1: No preincubation; expt. 2: preincubation in Ringer-Locke's solution without substrate for 2 hours; expt. 3: preincubation in Ringer-Locke's solution without substrate for 3 hours.
†$\mu l.O_2 \times 10^{-4}$ consumed per cell per hour.

demonstrated. On the contrary, in the presence of CO_2, acetate seemed to have an inhibiting effect, as shown in table 7.

If an increased formation of oxalacetate is responsible for the stimulating effect of CO_2 on cellular respiration, one would expect oxalacetate to have a similar effect. Furthermore one would expect that the effect of CO_2 would be less pronounced in the presence of oxalacetate, and vice versa one would expect less effect of oxalacetate in the presence of optimal concentrations of CO_2.

TABLE 7.—Effect of acetate and CO_2 on the respiration of Yoshida cells

Na-acetate concentration:		0 mM		1 mM		5 mM		Percent stimulation by acetate
Expt. No.	Gas phase	Respiration*	Percent stimulation	Respiration*	Percent stimulation	Respiration*	Percent stimulation	
1	Air	2.75		2.50				+9
	1% CO_2	2.98	8	1.55	+38			+48
2	Air	2.26				3.12		38
	1% CO_2	2.30	2			2.14	+31	+7

*$\mu l.$ $O_2 \times 10^{-6}$ consumed per cell per hour.

The results of 3 experiments carried out to elucidate the relationship between the CO_2 effect and oxalacetate are shown in table 8. Oxalacetate had a stimulatory effect on cellular respiration at room air, but less so at 2 percent CO_2. Furthermore the stimulatory effect of CO_2 was more pronounced in the absence of oxalacetate than in its presence. Thus in the present experiments CO_2 and oxalacetate could to a large extent replace one another.

TABLE 8.—Effect of oxalacetate and CO_2 on respiration of Yoshida ascites tumor cells*

Expt. No.	Oxalacetate concentration: (mM)	Oxygen consumption per cell per hour				Respiration stimulation by 2% CO_2 (% *increase*)
		0.03% CO_2		2% CO_2		
		($\mu l. \times 10^{+6}$)	(% *increase*)	($\mu l. \times 10^{+6}$)	(% *increase*)	
1	0	2.72	0	3.28	0	21
	1	3.02	11	3.05	+7	1
	10	3.82	40	3.78	15	+1
2	0	1.66	0	3.39	0	104
	1	2.05	24	3.62	7	77
	5	2.26	36	2.92	+14	29
3	0	2.57	0	5.20	0	102
	0.01	2.39	+7	5.69	9	138
	0.10	3.65	42			
	1.00	4.36	70	5.96	15	37
	10.00	5.53	115	3.72	+28	+33

*Experimental medium: Ringer-Locke's solution with 1 mM glucose.

DISCUSSION

The relationship which has been found between the effect of CO_2 on the respiratory metabolism and glucose, pyruvate, and oxalacetate supports the hypothesis that the CO_2 effect is due to an increased formation of oxalacetate.

The concentration of oxalacetate may be a rate-limiting factor in the respiration of cells, which contain the necessary enzymes and a surplus of active acetate. Due to the regeneration of oxalacetate in the citric acid cycle, a relative lack of oxalacetate is perhaps not likely to occur in

resting cells. In dividing cells, however, oxalacetate deficiency might occur partly due to the dilution after cell multiplication and partly due to the participation of oxalacetate in protein synthesis. This may explain why, in the present experiments, the greatest effect of CO_2 on the respiration of the Yoshida cells was found at a time when these cells showed the greatest mitotic activity.

The inhibitory effect of glucose on cellular respiration, first described by Crabtree (*14*), can be explained by a competition between the glycolytic and the respiratory system for adenosine diphosphate (*15–19*). This compound is required for 2 reactions of the glycolytic system, *viz.*, the conversion of 1,3-diphospho-D-glycerate to 3-phospho-D-glycerate, and of phospho-enol-pyruvate to pyruvate. Adenosine diphosphate, however, may be spared if phospho-enol-pyruvate reacts with CO_2 to form oxalacetate. In this process, which was described by Utter and Kurahashi (*12*), inosine diphosphate may function as phosphorus acceptor. This would explain the neutralizing effect of CO_2 on the Crabtree effect observed in the present experiments.

In our previous experiments, maximal respiration occurred at 0.5 to 2 percent CO_2 (*see* table 1). These findings indicate that phospho-enol-pyruvate plays a greater role than pyruvate for the CO_2 effect on cellular respiration. The effect of CO_2 concentration on the activity of phospho-enol-pyruvic carboxylase, which catalyzes the Utter and Kurahashi reaction, was studied by Walker and Brown (*20*). With an enzyme preparation obtained from Kalanchoe leaves, they found maximal activity at 0.25 to 3 percent CO_2. The malic enzyme reaction through which pyruvate is converted to malate by reductive carboxylation, as discovered by Ochoa, Mehler, and Kornberg (*13*), has a higher carbon dioxide requirement. As pointed out by Walker and Brown, Ochoa's figures (*21*) showed that the greatest activity occurred at the highest CO_2 concentration used (70%), whereas in the presence of 5 percent CO_2 this rate had fallen to about 27 percent of the maximum.

Whether a stimulation of the Utter and Kurahashi reaction can explain the growth-supporting effect of CO_2 in cell cultures remains to be shown. The demonstration of a growth-supporting effect of oxalacetate *in vitro* (*22*) indicates that this may be true. The relationship between the effect of CO_2 on cellular respiration and the mitotic activity shows that the rate of growth may be deciding for the role of CO_2/oxalacetate as an essential growth factor, which may explain the controversial findings of various authors (*2–4*). Of course the assumption that the growth-supporting effect of CO_2 is due to an increased formation of oxalacetate does not necessarily mean that growth stimulation is related to the higher rate of respiration. In the present investigation, no correlation was found between the mitotic activity and the rate of respiration, indicating that the function of oxalacetate as an amino acid precursor may be of greater importance for its growth-stimulating effect.

The Crabtree effect is assumed to be typical, though not specific, for malignant cells. The neutralizing effect of CO_2 on the Crabtree effect

indicates that this phenomenon may be of less significance under physiological conditions. However recent experiments in this laboratory (*23*) have shown that general conclusions cannot be drawn from the present experiments, since not all tumor cells respond to CO_2 in the same way as the Yoshida cells. It should also be remembered that, so far, no experiments have been carried out with CO_2 concentrations above 5 percent.

The ability of liver and the Murphy-Sturm lymphosarcoma to make dicarboxylic acids through CO_2 fixation *in vivo* was studied with isotopic techniques by Freedman and Graff (*24*), according to whom dicarboxylic acids are formed in tumor tissues through CO_2 fixation; but in contrast to liver, tumor tissue decarboxylates a significant portion of the dicarboxylic acids back to acetyl coenzyme A. These observations indicate that in some tumors respiratory stimulation by CO_2 may be due to a neutralization of the Crabtree effect alone, and not to an increase in the amounts of citric acid cycle metabolites.

Thus it seems that the Utter and Kurahashi reaction may have a different function in the metabolism of different tumor cells. Further studies may yield results that can be helpful in the evaluation of the evidence presented for and against the existence of respiratory lesions in neoplastic cells. The present investigation shows that studies carried out with manometric and isotopic techniques should take into consideration the influence of CO_2 on cellular respiration.

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Influence of the Mammary-Tumor Agent on the Genesis of Mammary Cancer in Agent-Free Mice After Male Transmission [1,2]

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SUMMARY

Spontaneous mammary cancer will develop in a considerable proportion of the breeding F_1 hybrids produced by mating strain C (BALB/c) females with strain C3H/An males, as determined by Andervont and Dunn and confirmed in this laboratory. The agent could not be demonstrated by bioassay of the tumors from old hybrids unless the mothers became infected and transferred the agent. The data to be considered are concerned with the influence of the mammary-tumor agent (MTA), transferred by males of various sublines of strain C3H, upon the etiology of tumors in F_1 offspring. Hybrids sired by males of strain C3H/AnBi and born in early litters to uninfected strain C mothers had a cancer incidence above 30 percent, while those with strain Z (C3H) fathers and born in later litters to uninfected mothers had an incidence of about 5 percent. The incidences were reversed when strain Z (C3H) males sired early litters and C3H/AnBi males the later ones. Fourteen tumors from hybrids averaging 589 days of age were tested by biological assay, including 1 tumor that appeared at 328 days. Three percent of the test animals had mammary cancer. After the strain C mothers became infected with the MTA, over 90 percent of their F_1 progeny developed mammary tumors at average ages less than 300 days, regardless of the subline of the male parent. At least 20 tumors from infected C mothers and their F_1 progeny were assayed in nearly 1,000 mice, of which 75 percent developed cancer. The tumor-inducing activity of these tumor extracts was higher at dilutions of 10^{-4} and 10^{-5} than at dilutions of 5×10^{-3} and 10^{-3}. There was no indication that the activity of the agent increased with the birth of successive litters. Various theories are discussed which deal with hormone-induced tumors in agent-free animals and the possible origin of the MTA; a suggestion is made of the role of the MTA in the genesis of mammary cancer in mice.—J. Nat. Cancer Inst. 25: 177–199, 1960.

INVESTIGATORS WHO have studied the genesis of spontaneous mammary cancer in mice agree that the mammary-tumor agent (MTA), with the properties of an infectious agent or virus, cannot be demonstrated by biological assay in all tumors of the mammary glands (*1–3*). The present material is concerned with the introduction of the MTA to agent-free mothers by male transmission and its influence upon the etiology of these so-called agent-free mammary tumors in the mothers and their

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progeny. Future publications will deal with data in which infection resulted from the administration of the agent in extracts of cancerous tissues.

Andervont (*4–7*) and Andervont and Dunn (*8–12*) found that the hybrids produced by mating agent-free strain C (BALB/c) females and males of strain C3H/An had a high incidence of mammary cancer. The average age at which cancer developed in the hybrids was at least 20 months, and usually the agent could not be demonstrated by bioassays of the tumors. They also determined (*12*) " . . . that the presence of the agent in the male does not exert a significant influence upon the occurrence of these tumors when the hybrids do not acquire the agent from the fathers." Few of the strain C females that they observed had mammary cancer and became infected with the agent from the males.

Although all investigators have reported a low incidence in agent-free females of the various sublines of strain C3H when they were continued as nonbreeders, a similar incidence has not been observed for breeders. Andervont and Dunn (*8*) found that only 3 percent of the agent-free breeding females of his strain C3H/An had tumors; in a subline descended from mice obtained from Andervont in 1941 Heston *et al.* (*13*) observed an incidence of 38 percent. In females used as either normal or forced breeders, Boot and Mühlbock (*14*) found incidences of 40 to 82 percent, and Deringer (*15*) noted incidences of 55 to 74 percent in strain C3H agent-free females of the Heston subline. Breeders of our agent-free subline of the C3H stock, called Zb, have had an incidence of 0.4 percent (*16, 17*).

MATERIALS AND METHODS

The maternal parents of the F_1 hybrids were of strain BALB/c, hereafter designated strain C, in accord with the recent practice of Snell (*18*), or "where the symbol is unwieldy, abbreviations are used."

The paternal animals were members of sublines of strain C3H and include males of our cancerous strain Z(C3H) and representatives of the Andervont subline of strain C3H, C3H/AnBi, inbred in this laboratory since 1948 for more than 20 generations. Other strain C3H/AnBi males were the descendants of females that had been foster-nursed by lactating females of our cancerous strain Z(C3H) to obtain the MTA of that cancerous stock. Males of our foster-nursed agent-free strain Z(C3H), or Zb line, also were used for some matings. The F_1 females were permitted to have as many litters as they would and they nursed their progeny.

The assay animals were ZBC hybrids (*19*), 23 to 27 days of age at the time of injection, with a tumor incidence of not more than 1 percent. To test for the MTA, 0.5 ml. of the supernatant was administered intraperitoneally at the dilutions (in gram equivalents) specified in the tables. The method of preparation of the extracts has been presented in other reports (*17, 20, 21*). In tabulating the assay mice, only noncancerous animals that survived for at least 300 days have been included. The mice received Purina laboratory chow and tap water.

EXPERIMENTAL RESULTS

Experiment I

Mothers.—Females of strain C were mated with males of our cancerous strain Z(C3H) for 4 or 5 litters, after which males of the agent-free Zb line were substituted.

Nineteen C females were continued, of which 7 (37%) developed mammary cancer; 6 remained uninfected and died without cancer, while the others died without cancer at 602 days of age, after being infected with the agent at either the fourth or fifth litter. The tumors in the cancerous females were first noted at an average age of 488 days; the uninfected females died at an average age of 558 days; these two groups averaged 8.4 and 8.2 litters, respectively. Five of the 7 cancerous females had become infected with the agent from the Z males by the time their third or fourth litters were born and the others, by the fifth or sixth litters, but in all instances the young from the litters born prior to infection of the mothers died at an early age and could not be continued.

Progeny.—Mammary cancer developed in less than 4 percent of the F_1 hybrids born to uninfected C females, whether the fathers were Z or Zb males, while those cast by infected mothers had an incidence of approximately 90 percent and an early average cancer age (table 1). In addition, the subline to which the fathers belonged did not influence the incidence in the progeny.

Tumor assay.—Six mammary tumors from F_1 hybrids were assayed for the MTA by the injection of extracts into agent-free ZBC test animals. One of these, a spontaneous tumor which appeared at only 439 days of age, arose in a hybrid born in the second litter of an uninfected C female. Administration of extracts of this tumor produced cancer in 1 of 40 mice. The other 5 tumors came from F_1 progeny of infected C mothers; 4 of the young had been sired by agent-free Zb males. Four percent of the mice are still living, yet 65 percent of the 217 mice that obtained extracts of the $CZbF_1$ tumors have had tumors, at an average age of 352 days, compared with 73 percent for the mice tested with extracts of the CZF_1 tumor (table 1). As may be seen from the data, the activity of the MTA was greater at dilutions of 10^{-3} and 10^{-4} than at 2×10^{-2}.

These data again demonstrate (*17*) that after females become infected with the MTA they will transfer the agent to their progeny, even to those sired by agent-free males, and that the MTA can be demonstrated by bioassay of the tumors.

Experiment II

Mothers.—Fourteen females were mated for 4 to 5 litters with males of the cancerous strain Z(C3H) and then to males of the cancerous strain C3H/AnBi. Four females developed mammary cancer at an average age of 476 days after transmission of the MTA by Z(C3H) males and 3 females, infected with the agent by C3H/AnBi males, had tumors at an average

TABLE 1

A.—Mammary cancer in F_1 progeny of females mated to cancerous Z(C3H) males for 4 to 5 litters, then to agent-free Zb males

F_1 hybrid	Number	With cancer (*percent*)	Average cancer age (*days*)
CZF_1			
Born to uninfected mothers	80	4	491
Born before infection of mothers	45	2	439
Born after infection of mothers	17	88	265
$CZbF_1$			
Born to uninfected mothers	59	4	491
Born to mothers that had been infected by Z males	49	100	284

B.—Biological assay for the MTA in ZBC mice

Donors			Assay data								
			2×10^{-2} Inoculum			10^{-3} Inoculum			10^{-4} or 10^{-5} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
CZF_1											
Born before infection of mother	1	439	22	0	—	20	5	285	—	—	—
Born after infection of mother	1	212	20	65	451	24	79	353	—	—	—
$CZbF_1$*	4	292†	87	54	353	92	68	335	38	79	388

*Preliminary data: 4 percent of the total number of assay mice for this group were still alive at 18 months of age.

†Average cancer age of group.

age of 642 days. The average number of litters borne by the cancerous females of the respective groups was 7.0 and 9.3. The females were infected by the Z males between the 4th and 6th litters and by the C3H/AnBi males, between the 5th and 8th litters. Two noncancerous C females had the MTA at the time their 5th litters were sired, one by a Z male, the other following the first litter by a C3H/AnBi male. Their respective ages at death were 597 and 553 days. One female remained noncancerous and uninfected; she died at 631 days of age after bearing 4 litters by a Z male and 5 litters by a male of strain C3H/AnBi. Four other females had an average of 5 litters by Z males, were not mated with C3H/AnBi males, and died without cancer at an average age of 453 days.

Progeny.—Details concerning the tumor data for the F_1 hybrids are given in table 2. Four percent of the 120 CZF_1 progeny born before the mothers were infected with the MTA had tumors at an average age of 543 days, but of the CZF_1 hybrids born to infected females, either cancerous or noncancerous, over 90 percent had tumors at an average age of 263 days. Observations on mice sired by C3H/AnBi mice are similar. When the mothers had the MTA, a high incidence and early average cancer age were noted in the F_1 offspring. Observations on small numbers of F_1 animals also indicate that those cast by females infected by C3H/AnBi males developed tumors earlier than the progeny of mothers infected by Z(C3H) males.

In 10 F_1 hybrids descended from 3 uninfected females and C3H/AnBi males, there was a cancer incidence of 80 percent, but their tumors did not develop until an average age of 634 days. Two of these mothers later became infected, and the 10 progeny born subsequently had an incidence of 100 percent and an average cancer age of 220 days.

Tumor assay.—As presented in table 2, 6 mammary tumors from infected F_1 hybrids were assayed in 338 test mice, of which 74 percent had tumors at an average age of 364 days. Of the total, 186 received dilutions ranging from 5×10^{-2} to 10^{-2}; they had an incidence of 65 percent and a cancer age of 386 days. The balance were injected with either 10^{-3} or 10^{-4} dilutions and had an incidence of 84 percent at 344 days.

Experiment III

Mothers.—Strain C females were mated with a group of males of the cancerous subline C3H/AnBi that had been foster-nursed by lactating females of the Z(C3H) cancerous line. Twenty-two females were used in this study, of which 5 died with cancer at an average age of 543 days and 15 remained uninfected and survived to an average age of 704 days. These 2 groups averaged 6.8 litters and 7.4 litters, respectively. The youngest female to have cancer was 333 days old and the oldest, 758 days. Infection of 3 of the cancerous females occurred between the 4th and 6th litters; no progeny were continued from late litters of the other 2 and so the exact litter at which they became infected cannot be stated. Two other strain C females had 9 and 8 litters of young, became infected

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progeny. Future publications will deal with data in which infection resulted from the administration of the agent in extracts of cancerous tissues.

Andervont (*4–7*) and Andervont and Dunn (*8–12*) found that the hybrids produced by mating agent-free strain C (BALB/c) females and males of strain C3H/An had a high incidence of mammary cancer. The average age at which cancer developed in the hybrids was at least 20 months, and usually the agent could not be demonstrated by bioassays of the tumors. They also determined (*12*) " . . . that the presence of the agent in the male does not exert a significant influence upon the occurrence of these tumors when the hybrids do not acquire the agent from the fathers." Few of the strain C females that they observed had mammary cancer and became infected with the agent from the males.

Although all investigators have reported a low incidence in agent-free females of the various sublines of strain C3H when they were continued as nonbreeders, a similar incidence has not been observed for breeders. Andervont and Dunn (*8*) found that only 3 percent of the agent-free breeding females of his strain C3H/An had tumors; in a subline descended from mice obtained from Andervont in 1941 Heston *et al.* (*13*) observed an incidence of 38 percent. In females used as either normal or forced breeders, Boot and Mühlbock (*14*) found incidences of 40 to 82 percent, and Deringer (*15*) noted incidences of 55 to 74 percent in strain C3H agent-free females of the Heston subline. Breeders of our agent-free subline of the C3H stock, called Zb, have had an incidence of 0.4 percent (*16, 17*).

MATERIALS AND METHODS

The maternal parents of the F_1 hybrids were of strain BALB/c, hereafter designated strain C, in accord with the recent practice of Snell (*18*), or "where the symbol is unwieldy, abbreviations are used."

The paternal animals were members of sublines of strain C3H and include males of our cancerous strain Z(C3H) and representatives of the Andervont subline of strain C3H, C3H/AnBi, inbred in this laboratory since 1948 for more than 20 generations. Other strain C3H/AnBi males were the descendants of females that had been foster-nursed by lactating females of our cancerous strain Z(C3H) to obtain the MTA of that cancerous stock. Males of our foster-nursed agent-free strain Z(C3H), or Zb line, also were used for some matings. The F_1 females were permitted to have as many litters as they would and they nursed their progeny.

The assay animals were ZBC hybrids (*19*), 23 to 27 days of age at the time of injection, with a tumor incidence of not more than 1 percent. To test for the MTA, 0.5 ml. of the supernatant was administered intraperitoneally at the dilutions (in gram equivalents) specified in the tables. The method of preparation of the extracts has been presented in other reports (*17, 20, 21*). In tabulating the assay mice, only noncancerous animals that survived for at least 300 days have been included. The mice received Purina laboratory chow and tap water.

EXPERIMENTAL RESULTS

Experiment I

Mothers.—Females of strain C were mated with males of our cancerous strain Z(C3H) for 4 or 5 litters, after which males of the agent-free Zb line were substituted.

Nineteen C females were continued, of which 7 (37%) developed mammary cancer; 6 remained uninfected and died without cancer, while the others died without cancer at 602 days of age, after being infected with the agent at either the fourth or fifth litter. The tumors in the cancerous females were first noted at an average age of 488 days; the uninfected females died at an average age of 558 days; these two groups averaged 8.4 and 8.2 litters, respectively. Five of the 7 cancerous females had become infected with the agent from the Z males by the time their third or fourth litters were born and the others, by the fifth or sixth litters, but in all instances the young from the litters born prior to infection of the mothers died at an early age and could not be continued.

Progeny.—Mammary cancer developed in less than 4 percent of the F_1 hybrids born to uninfected C females, whether the fathers were Z or Zb males, while those cast by infected mothers had an incidence of approximately 90 percent and an early average cancer age (table 1). In addition, the subline to which the fathers belonged did not influence the incidence in the progeny.

Tumor assay.—Six mammary tumors from F_1 hybrids were assayed for the MTA by the injection of extracts into agent-free ZBC test animals. One of these, a spontaneous tumor which appeared at only 439 days of age, arose in a hybrid born in the second litter of an uninfected C female. Administration of extracts of this tumor produced cancer in 1 of 40 mice. The other 5 tumors came from F_1 progeny of infected C mothers; 4 of the young had been sired by agent-free Zb males. Four percent of the mice are still living, yet 65 percent of the 217 mice that obtained extracts of the $CZbF_1$ tumors have had tumors, at an average age of 352 days, compared with 73 percent for the mice tested with extracts of the CZF_1 tumor (table 1). As may be seen from the data, the activity of the MTA was greater at dilutions of 10^{-3} and 10^{-4} than at 2×10^{-2}.

These data again demonstrate (*17*) that after females become infected with the MTA they will transfer the agent to their progeny, even to those sired by agent-free males, and that the MTA can be demonstrated by bioassay of the tumors.

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Mothers.—Fourteen females were mated for 4 to 5 litters with males of the cancerous strain Z(C3H) and then to males of the cancerous strain C3H/AnBi. Four females developed mammary cancer at an average age of 476 days after transmission of the MTA by Z(C3H) males and 3 females, infected with the agent by C3H/AnBi males, had tumors at an average

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Donors			Assay data								
			2×10^{-2} Inoculum			10^{-3} Inoculum			10^{-4} or 10^{-5} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
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*Preliminary data: 4 percent of the total number of assay mice for this group were still alive at 18 months of age.

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age of 642 days. The average number of litters borne by the cancerous females of the respective groups was 7.0 and 9.3. The females were infected by the Z males between the 4th and 6th litters and by the C3H/AnBi males, between the 5th and 8th litters. Two noncancerous C females had the MTA at the time their 5th litters were sired, one by a Z male, the other following the first litter by a C3H/AnBi male. Their respective ages at death were 597 and 553 days. One female remained noncancerous and uninfected; she died at 631 days of age after bearing 4 litters by a Z male and 5 litters by a male of strain C3H/AnBi. Four other females had an average of 5 litters by Z males, were not mated with C3H/AnBi males, and died without cancer at an average age of 453 days.

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In 10 F_1 hybrids descended from 3 uninfected females and C3H/AnBi males, there was a cancer incidence of 80 percent, but their tumors did not develop until an average age of 634 days. Two of these mothers later became infected, and the 10 progeny born subsequently had an incidence of 100 percent and an average cancer age of 220 days.

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Experiment III

Mothers.—Strain C females were mated with a group of males of the cancerous subline C3H/AnBi that had been foster-nursed by lactating females of the Z(C3H) cancerous line. Twenty-two females were used in this study, of which 5 died with cancer at an average age of 543 days and 15 remained uninfected and survived to an average age of 704 days. These 2 groups averaged 6.8 litters and 7.4 litters, respectively. The youngest female to have cancer was 333 days old and the oldest, 758 days. Infection of 3 of the cancerous females occurred between the 4th and 6th litters; no progeny were continued from late litters of the other 2 and so the exact litter at which they became infected cannot be stated. Two other strain C females had 9 and 8 litters of young, became infected

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TABLE 2

A.—Mammary cancer in F_1 progeny of females mated to cancerous Z(C3H) males for 4 or 5 litters, then to cancerous C3H/AnBi males

F_1 hybrid	Number	With cancer (*percent*)	Average cancer age (*days*)
(C × Z [C3H])F_1			
Born to uninfected mothers	31	3	321
	20	10	419
	5	0	—
	7	0	—
Born to mothers infected by Z(C3H)	57	4	543
	27	96	263
(C × C3H/AnBi)F_1			
Born to uninfected mothers	7	86	677
	0	—	—
	3	67	506
	0	—	—
Born to mothers infected by Z(C3H)	10	90	311
Born to mothers infected by C3H/AnBi	22	100	243
	8	75	325

B.—Biological assay for the MTA in the ZBC mice

Donors			Assay data								
			5×10^{-3}, 2×10^{-3}, or 10^{-3} Inoculum			10^{-3} Inoculum			10^{-4} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
(C × Z[C3H])F_1	1	203	28	61	391	26	73	345	25	88	381
(C × C3H/AnBi)F_1	3	242*	96	67	372	20	75	351	0	—	—
Mothers infected by Z(C3H)	3	195*	62	65	406	57	86	351	24	96	286

*Average cancer age of group.

after their fourth and fifth litters, and died without cancer at the age of 731 and 759 days, respectively.

Progeny.—As may be seen from table 3, the F_1 hybrids of C3H/AnBi males and uninfected mothers had an incidence in excess of 30 percent and developed cancer at an extremely late age. These data compare with an incidence of 31 percent at an average cancer age of 712 days for the progeny, numbering 101 hybrids, born in the first and second litters to the same mothers. As soon as the mothers became infected with the agent from males of the cancerous strain, the incidence in their F_1 offspring increased to about 90 percent, with an acceleration in the time of appearance of the tumors.

Tumor assay.—Eight tumors from F_1 progeny born to uninfected strain C mothers were assayed. The average age of the donors when their tumors were first observed was 616 days. The neoplasms were tested by injecting nearly 400 test animals with extracts of 2×10^{-2} to 10^{-4} dilutions. Three percent of the mice developed mammary tumors at an average age of 392 days. One of the mammary tumors had appeared in an F_1 hybrid from a first litter at 328 days of age. Extracts of this tumor were administered to 87 test animals but only 3 percent developed the disease, the same incidence observed in the entire group regardless of age of the donors.

Five mammary tumors from (C $\times$ C3H/AnBi)F_1 hybrids born to infected mothers were tested in approximately 200 mice. At the dilutions tested, no reduction in the activity of the agent present in the extracts was apparent; the incidence for the total number of mice was 88 percent, and the average age at cancer development was 321 days (table 3).

Experiment IV

Mothers.—Strain C females were mated with males of the cancerous C3H/AnBi line. This cross was repeated on the assumption that if an adequate number of animals are observed and produce a sufficient number of litters, some should become infected with the MTA by males of the cancerous strain.

Fourteen females survived for 300 days and 3 had mammary cancer at an average age of 500 days after bearing an average of 7.0 litters. One cancerous female became infected when her second litter was sired and another by her 7th litter; offspring born to the third cancerous female after her 2d litter, at which time she was still uninfected, were not continued.

Six of the 14 C females died without cancer at an average age of 667 days after bearing an average of 7.8 litters, but all had become infected with the MTA. Five of the females transferred the agent to the progeny born in their 6th to 8th litters, while the sixth female, which died at 648 days, was infected when her second litter was born. The last group of 5 females remained uninfected, had an average of 6.8 litters, and died at an average age of 665 days.

TABLE 3

A.—Mammary cancer in F_1 progeny of females mated to cancerous C3H/AnBi males foster-nursed by females of cancerous strain Z(C3H)

F_1 hybrid	Number	With cancer (*percent*)	Average cancer age (*days*)
Born to uninfected, noncancerous mothers	217	33	717
Born before infection of mothers			
Mothers later infected and cancerous	44	30	696
Mothers later infected but noncancerous	21	19	674
TOTAL	282	31	718
Born after infection of mothers			
Mothers cancerous	17	94	276
Mothers noncancerous	15	87	331
TOTAL	32	81	300

B.—Biological assay for the MTA in ZBC mice

Donors			Assay data								
			2×10^{-3} Inoculum			10^{-3} Inoculum			10^{-4} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
Born before infection of mothers	7	657*	137	2	226	144	3	449	26	0	—
Born in 1st litter before infection of mother	1	328	29	3	506	30	7	271	28	0	—
Born after infection of mothers	5	244*	82	82	339	99	92	314	21	95	295

*Average age of group.

Progeny.—Thirty-six percent of the F_1 females born to uninfected mothers, including those that never were infected and those that later became infected, developed mammary cancer at an average age of 687 days, whereas 97 percent of those born to infected mothers after they had become infected developed tumors at an average age of 255 days (table 4).

Tumor assays.—Three F_1 females born to uninfected C mothers had mammary cancer at an average age of 623 days. Their tumors were tested by injecting extracts of 5×10^{-2} to 10^{-3} dilutions into 89 ZBC mice. Mammary tumors developed in only 3 percent of the assay animals (table 4).

One tumor, from a strain C female infected when her second litter was born, had the MTA with considerable tumor-inducing activity—94 percent of the test animals developed cancer. The tumor was observed when the mouse was 483 days old, but no decrease in activity was apparent regardless of the dilution of the extracts. By chance, a tumor from a mouse born in the second litter was also tested; 30 ZBC females were inoculated with tumor extract, of which 70 percent died with cancer at an average age of 405 days.

Extracts of 5 mammary tumors from F_1 progeny of infected mothers were tested in 299 ZBC mice; 83 percent had tumors at an average of 343 days (table 4). Only 2 extracts were administered at 10^{-4} dilution and 1, tested in 24 of 38 mice, showed an incidence of only 67 percent compared with 86 percent for the other tumor. The respective incidences for all mice that received extracts of these 2 tumors, at all dilutions combined, were 69 percent and 85 percent.

Experiment V

Mothers.—Nine females were mated with males of cancerous strain C3H/AnBi for 3 to 6 litters, after which males of the cancerous strain Z(C3H) were used. None of the females were infected with the MTA by C3H/AnBi males. Three of the females developed mammary cancer at an average age of 667 days, had an average of 8 litters, and all became infected by the Z(C3H) males at their 6th litter. Three other females died without cancer at an average age of 694 days; 2 were infected with the agent at the birth of their 6th litters and the other by her 7th litter. They averaged 8 litters. The last 3 females died without cancer and uninfected at an average age of 668 days, after bearing an average of 9.3 litters.

Progeny.—Details concerning the development of mammary tumors in the F_1 hybrids of this study are presented in table 5, together with the assay data for the MTA. Of special interest was the cancer incidence in (uninfected C $\times$ Z[C3H])F_1 mice of 11 percent compared with 30 percent for (uninfected C $\times$ C3H/AnBi)F_1, and the increase to nearly 90 percent as soon as the mothers were infected by the Z(C3H) males.

Tumor assay.—Mammary tumors from 3 cancerous animals were tested by biological assay. One tumor developed in a female at 644 days

TABLE 4

A.—Mammary cancer in F_1 progeny of females mated to cancerous C3H/AnBi males

F_1 hybrid	Number	With cancer (*percent*)	Average cancer age (*days*)
Born to uninfected, noncancerous mothers	50	26	660
Born before infection of mothers			
Mothers later infected and cancerous	9	44	778
Mothers later infected but noncancerous	35	49	686
TOTAL	94	36	687
Born after infection of mothers			
Mothers cancerous	12	100	292
Mothers noncancerous	23	96	235
TOTAL	35	97	255

B.—Biological assay for MTA in ZBC mice

Donors			Assay data								
			5×10^{-2} or 10^{-2} Inoculum			10^{-3} Inoculum			10^{-4} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
Strain C female	1	483	29	90	364	25	96	326	25	96	316
F_1 born before infection of mothers	3	623*	45	2	566	44	5	476	0	—	—
F_1 born after infection of mothers	5	251*	136	82	356	125	86	332	38	74	330

*Average age of group.

TABLE 5

A.—Mammary cancer in F_1 progeny of females mated to cancerous C3H/AnBi males for 3 to 6 litters, then to cancerous Z(C3H) males

F_1 hybrid	Number	With cancer (*percent*)	Average cancer age (*days*)
(Uninfected C × C3H/AnBi) F_1	27	33	595
	20	25	694
TOTAL	47	30	631
(Uninfected C × Z[C3H]) F_1	27	15	665
	18	6	721
TOTAL	45	11	676
(Infected C × Z[C3H]) F_1	16	88	272
	0	—	—
TOTAL	16	88	272

B.—Biological assay for the MTA in ZBC mice

Donors			Assay data					
			2×10^{-2} Inoculum			10^{-3} Inoculum		
Mice	No.	Cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)	No. of mice	With cancer (*percent*)	Average cancer age (*days*)
Strain C mouse infected by Z♂ at 6th litter	1	644	16	88	326	19	89	292
(Uninfected C × C3H/AnBi) F_1	2	508*	38	0	—	40	3	580

* Average age of 2 mice.

of age and the other 2 tumors in F_1 hybrids when they were 484 and 531 days of age. Extracts of the F_1 tumors were tested in 78 mice, of which 1, an incidence of 1 percent, had cancer when 580 days of age. Extracts of the tumor from the cancerous strain C female were injected into 35 mice, of which 89 percent had mammary cancer at an average age of 307 days. There was no apparent decrease in the tumor-bearing activity at the dilutions tested, 2×10^{-2} and 10^{-3} (table 5).

The data presented in tables 1 to 5 are summarized in tables 6 to 8.

DISCUSSION

All investigators in the field of mammary-cancer genesis in mice are in general agreement that, etiologically, there may be more than one type of mammary disease. Those who have presented evidence for the development of agent-free mammary tumors in mice include Andervont, Bonser, Deringer, Dmochowski, Dunn, Foulds, Heston, Mühlbock, Passey, and the author (*see 1–3*). A few excerpts from various publications may be of value and interest in understanding the problem.

In a summary of the material presented at a recent symposium, Andervont (7) had this to say about the mammary-tumor virus: "For the

purpose of this discussion, however, the fact remains that the presence of the virus is not essential for the production of breast cancer in mice. Chemical carcinogens are also able to induce these tumors in strains of mice, that, under normal conditions, develop few breast tumors and do not harbor the virus."

Heston, Deringer, and Dunn (*22*) stated: "There is now ample evidence that not all mammary tumors of the mouse are associated with the agent . . . . The presence of the agent merely increases the probability that a tumor will occur and its absence decreases this probability. . . . There was no evidence at any time throughout the study that the agent remained inactive over a number of generations and then suddenly reappeared."

The lack of tumor-inducing activity in test animals after the administration of mammary-tumor extracts for biological assay has been interpreted as indicating the inability to demonstrate the presence of the MTA. It has been suggested by some, usually those who have not studied the disease in mice, that an agent was involved in the genesis of *all mammary tumors* in mice, even those which gave negative results following bioassays, due to an extremely low concentration of the "inciting agent." If this were the case, one would anticipate that some mammary tumors that developed in extremely old mice of cancerous strains also might possess an agent with similarly low tumor-inducing activity, which could be demonstrated only with difficulty. To the author's knowledge, such data have not been reported, and spontaneous mammary tumors from mice considered to harbor the agent have been found to have it when the tumors were tested by accepted methods. On the other hand, investigators have been in accord in reporting a lack of tumor-inducing activity only when tumors were assayed that developed in mice thought to be free of the MTA and, usually, in very old animals.

It might be recalled that several tumors have been studied in which the MTA could be demonstrated though the tumors had developed in mice considered to be agent-free (*17*, *23–25*). The cancerous animals were members of both susceptible (*17*, *23*) and resistant (*24*, *25*) strains. The female progeny and their mothers had not been injected with extracts containing the agent, nursed by females of cancerous stocks, or mated with males possessing the agent. The source of the agent in such tumors has not been determined.

One tumor was observed in a 17th-generation animal of the fostered Zb subline of strain Z(C3H); it was the only cancerous female found since the fostering of this line, which has a mammary-tumor incidence of 0.4 percent in breeding females (*16*, *17*). However, extracts of this tumor were as active in the production of tumors in test animals as were those from the average mammary cancer of high-cancer-strain donors (*17*).

Dmochowski (*2*) discussed the situation in 1958: "The M.T.V. does not appear to be involved in the origin of all mammary tumors; there are mammary tumors in which all attempts have so far failed to demonstrate it." And, "There are no essential morphological differences

between mammary cancer of virus-carrying and virus-free mice, although in the latter the tumors may show greater variation of structure."

"Different genetic mechanisms appear to be involved in the induction of mammary cancer in mice by carcinogenic hydrocarbons. They do not stimulate the production of a latent M.T.V., and the tumors may be of different type." When the tumors were induced in virus-free mice, the mammary-tumor virus (M.T.V.) could not be demonstrated by bioassay (reviewed in *2*).

At a recent symposium on mammary tumors, Foulds (*26*) considered the question of the presence or absence of the MTA in mammary tumors in mice as follows: "About this question of a latent virus; I have never believed it and I do not believe it now. I do not believe it has ever led to any useful information and I agree entirely with Dr. Mühlbock, Dr. Bonser, and Dr. Andervont, that the onus of proof is on the people who maintain that there is a virus there. I agree with Dr. Dmochowski that the failure to demonstrate the virus is not proof of its absence. But the people who maintain it is there have to demonstrate it. I think the assumption of a latent virus, which I heard a long time ago from another source, has never led to anything and I think it is dangerous."

To investigate the possible influence of the agent on the etiology of mammary tumor in hosts considered to be free of the MTA, it would be necessary to select mice of an inbred strain or hybrids of a generation in which mammary tumors are known to develop and where the MTA usually cannot be demonstrated by bioassay. By permitting groups of mothers to become infected with the agent at different times during the breeding period, one might evaluate any possible effect of the infecting agent upon the development of mammary cancer in both the mothers and their progeny. It should, in addition, be possible to demonstrate the presence of the agent by bioassay of extracts of the spontaneous tumors and to compare the tumor-inducing activities of various tumors, especially of those from offspring born to uninfected versus infected mothers. In the present studies the agent was transferred by male transmission, but other mice are under observation whose mothers received extracts containing the MTA in known concentration. In these mice it may be possible to obtain data on the development of cancer related to the amount of material injected in the mothers.

Andervont (*4–7*) and Andervont and Dunn (*8–12*) determined that a considerable proportion of (C[BALB/c]♀ × C3H/An♂)F_1 hybrids would develop mammary cancer at an average age of at least 20 months. Many tumors from extremely old F_1 donors were tested and failed to provide evidence of the presence of the agent. Except in cases where the hybrids acquired the agent from their fathers, the presence of the agent in the male did not exert any influence upon the development of tumors in the progeny (*12*).

Animals of the same inbred strains were mated in this laboratory. Forty-two percent of the breeding F_1 females had mammary tumors at an average age of 609 days (*16*). The incidence increased to 51 percent

when only noncancerous F_1 females that survived to the average cancer age were included. Of interest was the observation that the hybrids born in the first 2 litters had the same incidence as the offspring cast by the same uninfected mothers in later litters. Two of the 20 mothers died with cancer, and the incidence in their progeny was 29 percent compared with an incidence of 44 percent for the F_1 hybrids with noncancerous mothers.

Eight mammary tumors were assayed that developed in (C × C3H/AnBi)F_1 females when they were 512 to 651 days of age. Only 3 percent of the animals used for bioassay developed tumors; thus, the low tumor-inducing activity of these F_1 tumors is in accord with the accepted interpretation that the MTA could not be demonstrated.

After males of our subline of the cancerous strain Z(C3H) had been mated with noncancerous strain C females, 56 percent of the females developed mammary tumors and another 25 percent were infected with the MTA (*16*, *17*, *19–21*). Three females had cancer at 495 to 610 days of age. Extracts of their tumors were injected at dilutions of 2×10^{-2} and 10^{-3}, and the respective incidences in the test animals were 63 percent and 72 percent (*16*), due to the activity of the MTA transferred by males of the cancerous Z(C3H) stock to the females.

The CZF_1 hybrids born to uninfected females had an incidence of 5 percent and an average cancer age of 654 days, while infected mothers produced a total of 172 F_1 offspring, of which 93 percent had tumors at an average age of 254 days.

Biological assay of extracts of 4 spontaneous mammary tumors from infected CZF_1 hybrids showed the same tumor-inducing activity at dilutions of 10^{-3} or 10^{-4} as at 2×10^{-2} or 10^{-2}. Ten groups of test animals were observed, and the lowest incidence to be recorded in any group was 64 percent.

In these studies it was found that the agent-free females were infected with the MTA by males of the cancerous strains at the time of fertilization of any litter between the second and eighth, that females with amputated uterine horns could be infected by males regardless of their inability to bear offspring, and that vasectomized males also could transfer the agent to females. Variation was seen in the sensitivity to infection of females of agent-free but susceptible strains and in the ability of males of different cancerous strains to infect these females. Other data indicated that the source of the MTA carried by the male might be important. For example, males nursed by females of a different cancerous strain, so that they received the agent of the fostering stock, infected either more or fewer agent-free females than did unfostered males of the same strain, depending upon the strain of the foster mothers and the activity of the MTA (*16*, *17*, *19–21*).

The F_1 hybrids born in the first litters after the mothers became infected had the same incidence and average cancer age as did the progeny born to the same iufected mothers in later litters. These data indicate that there may not be any significant increase in the activity of the MTA

with the increasing age of the mothers and the birth of successive litters.

Other publications concerned with these problems are (*1, 8–12, 22, 25, 27–30; see 19* for recent review).

It was stated before that 56 percent of the strain C females developed mammary cancer when they were housed continuously with males of the cancerous strain Z(C3H) and another 25 percent became infected with the agent (*16–19*). Seventy-eight strain C females were observed in the present series, of which 32 percent had mammary cancer and 24 percent died without cancer but transferred the agent. The highest incidence, 37 percent, was seen when the females were mated with strain Z(C3H) males for 4 or 5 litters and then with agent-free Zb males. In addition, 32 percent of these females became infected but died without cancer. Thirty-six strain C females were mated with males of strain C3H/AnBi (experiments III and IV), 22 of these with males fostered by strain Z(C3H) females. Mammary tumors developed in 22 percent and the same percentage became infected but died without cancer. Because of controlled matings and the use of males of different sublines, one would hardly expect the same proportion of the females to have mammary cancer as when only males of cancerous strain Z(C3H) were mated without limit on the number of litters.

In the present series of experiments not more than 5 percent of the F_1 hybrids born to uninfected strain C mothers and fathers from the Z(C3H) subline of strain C3H had mammary cancer, but after C3H/AnBi males were substituted, the incidence in the (C × C3H/AnBi)F_1 hybrids of uninfected mothers increased to over 30 percent (table 6). Also, when the females were mated with strain C3H/AnBi for early litters and later with males of strain Z(C3H), the hybrids sired by males of the former subline had a higher incidence than did those with fathers of the latter (tables 2–6). These data demonstrate the importance of genetic differ-

TABLE 6.—Summary of spontaneous mammary-tumor data in F_1 hybrids born before and after infection of mothers with MTA by male transmission

Hybrids	Number	With cancer (*percent*)	Average cancer age (*days*)
(C × Z[C3H])F_1			
Born before infection	233	5	538
Born after infection	60	92	266
(C × C3H/AnBi)F_1			
Born before infection	151	37	665
Born after infection			
by Z(C3H)	10	90	311
by C3H/AnBi	65	95	258
(C × foster-nursed C3H/AnBi) F_1			
Born before infection	282	31	718
Born after infection	32	91	300
(C × C3H/AnBi) F_1—all groups			
Born before infection	433	33	697
Born after infection	107	93	275
(C × Zb) F_1			
Born before infection by Z	59	4	787
Born after infection by Z	49	100	284

ences transmitted by the males of the 2 sublines of the strain C3H to their progeny, which influenced the development of mammary cancer in animals where the MTA could not be demonstrated.

The hybrids with fathers from subline C3H/AnBi had an incidence of 37 percent, compared with an incidence of 31 percent for those with fathers from the same subline that were fostered by strain Z(C3H) females (table 6). In a sample of 101 hybrids with foster-nursed strain C3H/AnBi fathers, those born in the first 2 litters had an incidence of 26 percent while those cast in later litters showed an incidence of 32 percent. In every group of hybrids born to uninfected females, regardless of the subline of the fathers, the average cancer age was 20 to 25 months (table 6).

Mention should be made of the small number of (C × C3H/AnBi)F_1 females with an extremely high incidence of mammary tumors, described in table 2. These hybrids were born to 3 females that had remained uninfected after mating with strain Z(C3H) males for 4 or 5 litters and then with males of strain C3H/AnBi. These mothers had 21 CZF_1 offspring and eventually all died without cancer after being used as breeders. The same 3 females had 10 F_1 progeny fathered by strain C3H/AnBi males; 8 of this group had mammary cancer at an average age of 634 days. Although no tumors from these cancerous (C × C3H/AnBi)F_1 hybrids were assayed for the MTA, the late cancer age is in accord with other data indicating that these females did not have the agent. Two of the mothers later were infected with the MTA by strain C3H/AnBi males, while the third remained uninfected. The 2 mothers had 10 F_1 progeny after they became infected with the agent; all died with cancer at an average cancer age of 220 days.

Spontaneous mammary tumors from 14 F_1 females with uninfected mothers were assayed. These hybrids had fathers from different lines of strain C3H and had an average cancer age of 589 days. As only 3 percent of the assay mice developed tumors, the data did not indicate the presence of the MTA in any tumor (table 7). One of the cancerous F_1 females was born in the first litter and developed a tumor at only 328 days of age. Extracts of this tumor were tested at various dilutions in 87 mice, of which only 3 percent showed the disease (table 3), the same incidence as that for tumors from older hybrids.

As soon as mothers became infected with the MTA from males of any cancerous C3H subline, the cancer incidence in their F_1 progeny increased to over 90 percent, together with a corresponding acceleration in the average time of appearance of the tumors. The number of mice observed in the various groups was not adequate to show whether the source of the agent had any significant influence upon the cancer age in the F_1 progeny. There was an indication, however, that among the hybrids sired by C3H/AnBi males, those born to females infected by strain Z(C3H) males developed tumors at a later age than those born to females infected by strain C3H/AnBi males (table 6).

Twenty F_1 females had mammary tumors at an average age of 242 days, and over 1,000 mice were used in bioassays of their tumors. These

TABLE 7.—Summary of data on biological assay for MTA at all dilutions

Tumor source	Number of tumors	Average age of donors (*days*)	Assay data		
			Number of mice	With cancer (*percent*)	Average cancer age (*days*)
F_1 hybrids of uninfected mothers					
(C × Z[C3H])F_1	1	439	42	2.4	285
(C × C3H/AnBi)F_1	5	577	167	2.9	525
(C × foster-nursed C3H/AnBi)F_1	8	616	394	2.5	392
TOTAL	14	589	603	2.7	427
F_1 hybrids of infected mothers					
(C × Z[C3H])F_1	2	208	123	73	379
(C × C3H/AnBi)F_1	7	222	442	81	360
Mothers infected by Z	2	242	116	68	368
Mothers infected by foster-nursed C3H/AnBi	5	244	202	88	321
(C × Zb)F_1—mothers infected by Z	4	292	217	65	352
Infected strain C mice					
Infected by C3H/AnBi at 2d litter	1	483	79	94	336
Infected by Z(C3H) at 6th litter	1	644	35	89	307

hybrids were born to infected mothers and are listed according to the subline of the father (table 7). The lowest mean incidence recorded for any group of tumors was 65 percent and the highest 88 percent. The latter figure was found in hybrids with strain C3H/AnBi fathers that were foster-nursed by females of strain Z(C3H). If, as indicated in table 6, the source of the MTA influenced the time of development of spontaneous tumors in the F_1 hybrids, there was no evidence that it had any effect upon the tumor-inducing activity of the agent extracted from these tumors and tested by biological assays.

Tumors from $CZbF_1$ and CZF_1 hybrids showed approximately the same activity. The MTA, in females of both groups, was transferred by males of the cancerous strain Z(C3H), to the mothers, who then transferred it via the milk, to their hybrid progeny (table 7).

Two tumors from infected mothers showed exceptional tumor-inducing activity—90 percent of the ZBC assay mice developed cancer. One female did not become infected by a strain Z(C3H) male until her sixth litter and the tumor did not develop until the female was 644 days of age. Extracts of this tumor showed equal activity at dilutions of 10^{-3} and 2×10^{-2} (table 5).

The assay data for tumors from F_1 females with infected mothers are summarized in table 8, according to the dilution of the extracts. Nearly 1,000 ZBC mice were used in these studies, of which 75 percent developed mammary cancer at a mean cancer age of 351 days after the injection of the MTA-containing extracts. The highest incidences were observed after the injection of tumor extracts at dilutions of 10^{-3} to 10^{-5} rather

than at the more concentrated dilutions of 5×10^{-2} to 10^{-2}. The mean incidences for these respective groups were 81 and 68 percent and the average cancer ages, 338 and 369 days.

TABLE 8.—Summary of data on biological assay of tumors from F_1 hybrids of infected mothers

Dilution of inoculum (*gm. equiv.*)	Number of mice	With cancer (*percent*)	Average cancer age (*days*)
5×10^{-2}	37	68	317
2×10^{-2}	308	69	372
10^{-2}	102	65	378
10^{-3}	387	80	338
10^{-4}	127	86	334
10^{-5}	19	74	383

Although the figures indicated a lower activity for the extract injected at a dilution of 10^{-5}, only 19 mice received this dilution, while 43 mice were tested at dilutions of 2×10^{-2} and 10^{-3}. The incidence in the mice that received the more concentrated doses was 56 percent, compared with 74 percent when the same extract was tested at 10^{-5} dilution.

Since less than 3 percent of the test animals developed tumors when they received injections of tumor extracts from hybrids born to uninfected mothers, these data have not been summarized.

Mühlbock and Boot (*31–34*) found that weekly subcutaneous transplantation of hypophyses, in some cases for a year or longer, caused the development of mammary cancer in agent-free females maintained as virgins. To date (*33*), mammary tumors have been induced in nonbreeding females of 6 inbred strains and 2 hybrid groups lacking the MTA, with incidences in some groups as high as 100 percent. Donors were mice of the same agent-free strain as the recipients. In no instance could the mammary-tumor agent be detected by biological assay.

The authors concluded that these mammary tumors must be considered purely hormonally induced, with prolactin thought to be the active hormone. According to Boot and Mühlbock (*34*), preliminary data from other experiments indicate that transplants in the spleen, kidney, or ovary may be more active than subcutaneous implants, and mammary cancer can be induced by fewer grafts. Mühlbock (*32*) explained the development of mammary cancer thus: "This experience led us to the assumption that the hormonal factor is a primary one in the development of mammary cancer and that the mammary tumor agent is an accelerator or intensifier."

The idea that the agent may act as an accelerator had been suggested previously by Andervont and Dunn (*8*). According to their theory, the role of the agent in the genesis of mammary cancer would be comparable to that of carcinogens in the induction of pulmonary tumors in mice.

The observations of Mühlbock and Boot have been corroborated in this laboratory by Halberg *et al.* (*35, 36*), who found that mammary cancer

could be induced with implants of pituitary glands in agent-free females of strain C and accelerated in females of the cancerous strain D_8, and that the incidence and cancer age in females of both groups was influenced by the number of implants. In pituitary-isografted strain C mice there was persistence of the adrenal cycle and alteration of the estrous cycle. Insertion of the hypothalamus at the time of pituitary implantation delayed mammary carcinogenesis by mechanisms as yet unknown.

In mice of susceptible stocks with the MTA there is desynchronization of 24-hour periodicity with an altered mitotic rhythm (Halberg and Bittner, unpublished data). This possible aspect of "carcinogenesis" may be detected shortly before the animals attain the cancer age. These associations are not completely understood at present, but it may be that the agent is acting in some manner through the hormonal mechanism of the host, with the altered hormones becoming the primary inciter in the development of spontaneous mammary cancer.

It is now known that inherited hormonal patterns may either "induce" or "inhibit" the development of mammary cancer in mice known to possess all other causative factors for the spontaneous disease (*2, 37*).

Dmochowski (*1*) found that the behavior of the agent *in vivo* differed from that of other known viruses and in 1958 (*2*) stated: "The M.T.V. as yet has not been separated from normal tissue components and there is no convincing proof of specific antigenic differences between the M.T.V. and tissues from which it originated." The following quotation may be cited, as it considers the possible origin of the mammary-tumor agent or virus, which would perhaps account for some of the above-mentioned properties:

"The mammary tumor-inducing agent has been compared with a cytoplasmic constituent which may have arisen by a transformation of a normal cell constituent (*see* Dmochowski, 1953a). The agent has also been compared with viral agents arising by mutation of cytoplasmic determinants, plasmagenes and the cytoplasmic 'Kappa' factor of paramecia (Dmochowski, 1953a). The value of this comparison is that it stresses the necessity of looking at the agent not as an entity in itself, but as an entity among the physiologically coordinated nuclear and cytoplasmic units which comprise the cell." (*25*). The 1953a citation in the quotation is our reference (*1*). A similar theory had been suggested earlier by Heston (*38*).

According to Dmochowski (*2*) and Dmochowski and Grey (*39–41*), the relationship has not been established between the viruslike particles, seen with the electron microscope, and the results of biological assays of mammary tumors containing the particles. They stated that further work was required to establish the nature of the particles, since "Similar particles were found, however, in the microsomal fraction devoid of any tumor-inducing activity, and were obtained from spontaneous mammary tumors of mice from strains apparently free of the virus." (*40*).

In neutralization studies it was determined that antiserum elicited against normal mammary tissue containing the MTA was as effective in

neutralizing the MTA as was antiserum prepared in either rabbits or guinea pigs against cancerous tissue, while that produced against agent-free normal tissue had no apparent effect (*20*). Also, an antigenic variation was noted between the MTA of females of strain A and that of their $(A \times Z)F_1$ progeny. Even though the hybrids had been nursed by their strain A mothers, from whom they obtained the MTA, the antiserum produced against the agent from the F_1 hybrid was ineffective, either *in vitro* or *in vivo*, in neutralizing the agent from the maternal strain, though it was active against the agent from the F_1 hybrids (*42*).

These observations have been extended by transplanting spontaneous mammary tumors in mice of the inbred strains and their agent-free F_1 hybrids and testing the antiserums elicited against the agent carried in the transplanted tumors. The observations from this study follow (*20*):

"The antisera produced against the MTA from tumors grown in inbred mice neutralized the agent in extracts of the same tumors inoculated in either inbred or hybrid animals, while the antisera elicited against the tumors from F_1 hosts were active against the agent from the same tumors transplanted in F_1 hybrids but not against the agent from the same tumor carried in inbred animals.

"The experimental data indicate that the antigenic characteristics of the mouse mammary tumor agent may be influenced by the incorporation of normal tissue component(s) of the host in the virus particles.

"In one experiment, the antiserum produced against the A tumor transplanted in F_1 hybrids for five passages reduced the tumor-inducing activity of the MTA obtained from the Z tumor of the 11th passage also from F_1 hosts, while the A in A tumor antiserum was inactive.

"Observations from another preliminary study indicate that, while the MTA present in the Z tumor following inoculation in the $(A \times Zb)F_1$ mice had become antigenically different from the agent extracted from the Z tumor transplanted in Z mice, after the Z tumor from F_1 hosts was again passed in Z mice for several passages, the agent in the tumors had regained the antigenic characteristics of the agent in the Z tumor from Z mice.

"The data presented in this report on neutralization studies of the MTA would suggest that some normal tissue component(s) contributed by the host had influenced the antigenic characteristics of the agent, in accord with the observations reported previously. . . ." (*20*).

Although the mouse mammary-tumor agent may not be antigenic in mice (*see* reviews *2* and *43*), differences have been demonstrated between antiserums prepared in rabbits and guinea pigs against mouse tissues containing or free of the MTA in neutralization tests (*20*, *44*). In addition, it has been possible to distinguish antigenically between the agent transferred by females of a cancerous inbred strain and that extracted from tissues of their F_1 progeny. This could be explained on the basis that normal tissue component(s) had been contributed by the host and incorporated in the agent particles, influencing their antigenic characteristics. Since the genetic constitution of animals of an inbred strain is quite different from that of their F_1 hybrids, comparable variations would

exist in the tissue material that might become part of the agent molecules and determine the properties of the MTA.

These observations would support the theory suggested by Heston (*38*) and Dmochowski (*1, 25*) that the mammary-tumor agent may have resulted from a mutation or transformation of a normal cell constituent.

Perhaps at this time it would be of interest to cite Stanley (*45*), who stated, ". . . it would appear wise to revise the generally accepted definition of a virus to include nucleic acids and perhaps also to include replicating structures which do not evidence infectively in the usual sense. . . . Actually, the idea that nucleic acid might possess activity is not new since genes have sometimes been regarded as nucleic acid and the actions of genes and viruses have long been regarded as being similar in quality," for, according to Lederberg (*44*): "We now have the means to deal with viruses as 'genes' which function at the parasitic level."

While it is of major importance to identify the mouse mammary-tumor agent or virus, equal effort should be devoted to investigations which may increase our knowledge of the role of the MTA in the etiology of mammary cancer in mice. Andervont (*7*) has stated that studies on the agent have emphasized the complexity of this virus-induced tumor and that the biological characteristics of the agent may not be as important as its contribution to our information about cancer viruses.

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Modification of Growth and Function of a Transplanted Ovarian Granulosa-Cell Tumor[1,2]

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SUMMARY

A granulosa-cell tumor was transplanted into 375 hybrid mice. Production of estrogen by the tumor was revealed in female mice by maintenance of uterine weight in ovariectomized hosts and by the development of polycystic uteri in intact mice. Hormone production was indicated in male mice by the concurrent atrophy of testicular and seminal vesicles, development of the mammary glands, and formation of an interpubic ligament. The uterine stromal nuclei resembled those in mice that received progesterone, which indicated that a progesterone-like hormone was also produced by the tumor. Growth rate of the tumor in male hosts was increased by progression of tumor generation and decreased by administration of thyroid and of thiouracil after orchiectomy, hypophysectomy, and testosterone-pellet implantation and injection of estradiol benzoate. Tumor growth in female mice was increased by progression of tumor generation, castration, and testosterone treatment and decreased by injection of estradiol benzoate. The latent period of tumor development was shorter in male mice than in female mice. Hormone production of the tumor decreased with progression of tumor generation, and increased following transplantation into a 2-day-old mouse. In male hosts hormone production of the tumor increased during the administration of thyroid, thiouracil, and a combination of FSH, LH, and LTH. Changes in the endocrine environment of the host had no detectable effect on the hormone production of tumors transplanted into female mice. Tumors that developed in thyroid-treated male mice showed increased sudanophilia. Otherwise treatment of the hosts did not alter significantly the histological characteristics of the tumors that were studied. An increase in the level of LH or a change in the responsiveness to LH was postulated as the mechanism of action of thyroid and thiouracil. The increased latent period of tumor development in female mice may be due to the higher levels of LH in females as compared to male mice. Growth and function of the transplanted granulosa-cell tumor could be modified independently of each other.—J. Nat. Cancer Inst. 25: 201–216, 1960.

THE ENDOCRINE environment seems to influence both the experimental induction of ovarian tumors and the growth and function of these tumors following transplantation (*1–3*).

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The rate of growth and hormone production of transplanted ovarian tumors have changed during successive subpassage, by differences in sites of transplantation, sex, and hormonal or operative treatment of the hosts. Successive transplantation of tumors had no predictable effect on the percentage of tumor takes; the percentage of takes increased with the subpassage of one line of granulosa-cell tumor and decreased with the subpassage of a second line of granulosa-cell tumor (*3, 4*). Subpassage of granulosa-cell tumors may increase or decrease the hormone production of the tumor with or without concomitant morphological changes, except that tumors no longer produced estrogen after a sarcomatoid transformation (*2, 5*). Male hosts usually afforded the most favorable environment for the growth and hormone production of granulosa-cell tumors (*3, 6*). Different granulosa-cell tumors show significantly different responses to the same environments to which they are subjected; some grow more rapidly in male than female and in intact than in castrated hosts. Each tumor tends to show specific growth characteristcs (*2, 3*).

No correlation existed between hormone dependence and estrogen secretion of 19 granulosa-cell tumors (*7*). The present study was undertaken to determine whether the growth and function of a single granulosa-cell tumor could be modified independently by changes in the endocrine environment of the host.

MATERIALS AND METHODS

Of the 411 hybrid mice (A × C57) used in this experiment, 159 were females and 252 were males. The mice were born and raised in an air-conditioned room adjusted to 72 to 74° F. After they were weaned at approximately 20 to 25 days of age they were maintained on Purina laboratory chow (Ralston Purina Company, St. Louis, Mo.) and water *ad libitum*. The mice were vaccinated against ectromelia with smallpox vaccine [V-5 smallpox vaccine (vaccine virus calf lymph) Eli Lilly & Company, Indianapolis, Ind.] when they reached approximately 40 days of age. At the time the mice received grafts of tumors, or when they were selected as recipients of such transplants, female and male mice were separated. Mice from the same litter were usually placed into 2 or more different groups.

The ovarian granulosa-cell tumor used originated in the right ovary of an A71 (A × C57) mouse that received 175 r total-body irradiation at 86 days of age. The ovarian tumor was pale yellow and microscopically was follicular in type. The granulosa cells were arranged in small follicles or around small cystic areas and sinusoids. Connective-tissue stroma was scant; vascular sinusoids were abundant. The tumor gave evidence of estrogen production (*8*). A portion of the tumor was cut into pieces, approximately 1 to 2 mm. in diameter, that were introduced subcutaneously through a #13 trocar in the right inguinal region of the hosts. The first transplantation generation shall be designated T1 and successive generations T2 to T6.

Donor mice from T1 to T5 were males and were killed by cervical fracture. Animals selected for hosts were 40 to 220 days old—usually 60 to 140 days old. Two transplants were made into hosts 2 to 3 days of age. Animals were palpated to detect growth of the grafts 2 to 4 weeks after transplantation and subsequently at weekly intervals. When tumors were palpable, they were measured with a vernier caliper. The length (longest measurement) and the width (a measure at approximately 90° to the length) were recorded.

Animals were killed either when the greatest diameter of the tumor measured 15 mm., or greater, or when an experiment was arbitrarily terminated at least 4 months after tumor transplantation. Animals in which tumors did not appear were killed 4 to 12 months after tumor transplantation. At necropsy, the liver, adrenal glands, submaxillary glands, kidneys, spleen, uterus, and ovaries, or testes and seminal vesicles, were weighed. The thorax and pituitary gland were inspected. The length of the interpubic ligament was measured. Mammary glands of the left side of male mice were fixed in Bouin's solution and stained in Mayer's hemalum. Two representative pieces of tumor were saved, one in 10 percent formalin, the other in Bouin's solution. Any abnormal organ, the adrenal glands, uterus, and ovaries or testes were fixed in Bouin's solution, sectioned at 7 μ and stained with hematoxylin and eosin. Tumors fixed in 10 percent formalin were embedded in 10 to 15 percent gelatin and cut with a freezing microtome at 12 μ. Representative tumors were stained with Sudan IV and counterstained with hematoxylin. Other sections were stained with periodic acid-Schiff's reagent [4] and counterstained with Delafield's hematoxylin.

The following dosage schedules and materials were used:

1 Armour unit of follicle-stimulating hormone (FSH) [5] in 0.05 cc. of saline every other day; equine gonadotropin (FSH and LH) [6]

0.5 mg. prolactin (LTH) [5] containing 20 to 25 IU per mg.

A solution containing 0.5 AU of FSH plus 0.5 mg. of equine gonadotropin plus 0.5 mg. LTH in 0.5 cc. saline injected every other day

16.6 μg. estradiol benzoate [6] in 0.05 cc. of sesame oil, and 33.2 μg. per 0.05 cc. injected weekly

1.25 mg. testosterone propionate [7] in 0.05 cc. of sesame oil, of which 0.02 cc. were injected in female mice at 1, 7, and 14 days of age

Testosterone pellets,[6] weighing 14 mg., implanted subcutaneously

50 μg. cortisone acetate [8] in 0.05 cc. saline 3 times weekly

2 gm. desiccated thyroid extract [5] added to 1 kg. powdered Purina laboratory chow to make a 0.02 percent mixture

10 mg. thiouracil [9] added to 1 kg. powdered Purina laboratory chow to make a 1 percent mixture

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[4] Schiff's solution according to Coleman and Glick: Techniques of Histochemistry and Cytochemistry. New York, Interscience Publishers, 1949, p. 67.

[5] Supplied by Armour Laboratories, Chicago, Ill.

[6] Supplied by Ciba Pharmaceutical Products Inc., Summit, N.J.

[7] Testosterone propionate with benzyl alcohol 10 percent and propylparaben 0.1 percent added as preservative, supplied as Oreton propionate by Schering Corporation, Bloomfield, N.J.

[8] Supplied by Merck & Company, Inc., Rahway, N.J.

[9] Supplied by Mann Research Laboratories, Inc., New York, N.Y.

Operative treatments consisted of ovariectomy, orchiectomy, orchiectomy with an ovarian transplant subcutaneously, and hypophysectomy.

Most of the experimental animals had tumors of the fifth transplant generation (T5). Five T4 donor animals (#1, 2, 3, 8, and 15B—15B was 2 days of age when it received its tumor transplant) and one T5 donor animal, #205, were used. From 12 to 121 mice received transplants from any one donor. Each treatment group had 5 to 10 mice. Within each transplantation group 5 to 12 animals were used as controls and received no treatment following tumor transplantation. When a group showed significant differences in tumor growth or hormone production, the experiment was repeated with another group of animals. One experimental group of 36 male mice did not receive tumor transplants but served as a control for the groups of mice that received injections of hormone.

The growth of a transplanted granulosa-cell tumor and its response to changes in the endocrine environment of the host were studied by determining: (1) the percentage of grafts that grew, (2) the latent period between the day of transplantation and the day the graft became palpable, and (3) the rate of growth of the graft—the period of time required for the palpable transplants to attain an area of 150 mm.^{2} The hormone production of the tumor in female mice was indicated by atrophy of the ovaries, maintenance of uterine weight in castrated females, and development of polycystic uteri in intact females. Hormone production of the tumor in male mice was indicated by atrophy of testicular and seminal vesicles, mobility of the interpubic joint, development of inguinal hernias, and ductal and alveolar development of the mammary glands. Each animal was used for the bioassy of its own tumor.

Influences which might effect the growth and hormone production of the tumor include: (1) generation of the tumor, (2) sex of the host, (3) age of the donor animal when tumor was implanted, and (4) the hormonal environment in the host as influenced by operative procedures or administration of extrinsic hormones.

OBSERVATIONS

Hormone Production, Rate and Incidence of Growth of Transplanted Tumors

The latent period and estrogenic activity of the transplanted tumor decreased with subpassages as manifested by decreased uterine weight and testicular atrophy (tables 1 and 2). No "significant" [10] change in the growth period occurred between T5 and T6 mice. The morphology of the tumor was unchanged between T1 and T6 generations.

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[10] "Significant" is used when P values calculated either from straight T or Chi square are less than 0.05.

TABLE 1.—Comparison of percentage of tumor takes, latent period, and growth period in T1 to T6 tumor-transplant generations in male and female mice

Tumor generation	Number and sex	Percent of takes	Latent period (*months*)	Growth period (*months*)
T1	5♂	60	—	—
T1	7♀	71	—	—
T2	19♂	42*	—	—
T2	24♀	58*	—	—
T3	29♂	83	—	—
T3	29♀	86	—	—
T4	20♂	95	5.5† (4 mice)	
T5	43♂	98	1.5† (20 mice)	2.2 (20 mice)
T5	36♀	83	4.0† (16 mice)	3.0 (15 mice)
T6	19♂	84	1.3† (16 mice)	1.6 (9 mice)
T6	7♀	100	2.0† (7 mice)	2.3 (6 mice)

*Difference between T2♀ and T2♂ and T3, T4, T5, and T6 generations: $P = <0.01$.
†Difference in latent period: $P = <0.01$ between T4 and T5♂ mice; T5 and T6♀ mice; T5♂ and T5♀; and T6♂ and T6♀.

TABLE 2.—Comparison of hormone production in male and female mice bearing granulosa-cell tumors weighing 1 gm. or more from T5 and T6 generation

Tumor generation	Number and sex	Donor	Mean uterine weight (*mg.*)	Testes mean weight (*mg.*)	Seminal vesicles mean weight (*mg.*)	Tumor mean weight (*gm.*)
T5	10♀	1, 3, 8	170*	—	—	2.4
T6	6♀	205	114*	—	—	2.2
T5	13♂	1, 3, 8	—	127*	37†	3.1
T6	9♂	205	—	160*	27	3.1

*Difference between uterine weight T5 and T6 female, and testicular weight T5 and T6 male: $P = <0.05$.
†Difference between seminal-vesicle weight T5 and T6 male: $P = > 0.1$.

Sex of host.—The average latent period among the male mice bearing tumors of T5 and T6 generation was significantly less than among the female mice. The sex of the host had no significant effect on either the percentage of takes or the growth period (table 1). The relative hormone production of the tumor in male and female mice cannot be compared.

Age of donor animal at time tumor implanted.—The tumors that developed in mice with transplants from a donor animal 2 days of age at the time of transplantation had a decreased latent period and an increased estrogenic activity as compared to tumors that developed in mice with transplants from donors 90 and 97 days old at the time of transplantation. The testes were smaller and uteri larger in the male and female hosts bearing tumors from young hosts. Age of the donor animal had no significant effect on the percent takes or the growth rate of the tumor (table 3).

Hormonal Environment of Host

Thyroid and Thiouracil

Both the growth and function of the tumor was influenced in thyroid-fed animals that received transplants from 3 donor animals. In group 1 (donor 8 T4) 20 percent of the transplanted tumors grew as compared to

TABLE 3.—Comparison of percentage of takes, latent period, growth period, and hormone production of granulosa-cell tumors in male and female mice that received transplants from donor animals 2, 90, and 97 days of age at time of transplantation

Age of donor (*days*)	Number and sex	Percent of takes	Mean latent period (*weeks*)	Mean growth period (*months*)	Mean testes weight (*mg.*)	Seminal vesicles (*mg.*)	Mean tumor (*gm.*)
2	6♂	100	4	3	82	14	2.0
90–97	13♂	100	6½*	2†	127*	37†	3.1
					Mean uterine weight (*mg.*)		
2	5♀	80	3½	3	250		1.9
90–97	25♀	80	16‡	3	170*		2.4

*Difference between 2 day and 90 and 97 day: $P<0.05$.
†Difference between 2 day and 90 and 97 day: $P>0.1$.
‡Difference between 2 day and 90 and 97 day: $P<0.01$.

control animals with 100 percent tumor takes. In group 2 (donor 15B T4) the latent period and extent of testicular atrophy were increased. In group 3 (donor 205 T5) the latent period and growth period were both increased. The hyperthyroid mice seemed to support less rapid growth of the tumors and an increased estrogen production (table 4).

TABLE 4.—Effect of thyroid and thiouracil in male mice on percentage of tumor takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*weeks*)	Mean growth period (*months*)	Mean testes weight (*mg.*)	Seminal vesicles mean weight (*mg.*)	Mean tumor weight (*gm.*)
Thyroid	8 T4	5	20*	—	—	—	—	—
Control	8 T4	7	100	7	1	136	98	0.9
Thyroid	15B T4	6	100	6*	3	42†	13	1.3
Control	15B T4	6	100	4	3	81	14	2.0
Thyroid	205 T5	6	100	11*	2½†	124	81	1.0
Control	205 T5	12	83	6	1½	160	27	3.3
Thiouracil	8 T4	6	83	17*	2†	115†	17*	1.5
Control	8 T4	7	100	7	1	136	98	0.9

*$P < 0.01$.
†$P < 0.05$.

One group of male mice (donor 8 T4) received 1 percent thiouracil. These animals had a longer latent and growth period and smaller testes and seminal vesicles than control animals (donor 8 T4) that received a normal diet (table 4).

The testes of thyroid-treated mice were usually small and revealed variable edema, necrosis or atrophy of spermatic tubular cells, and interstitial cells. Thyroid-treated mice showed more testicular edema and necrosis and less spermatogenesis than controls bearing tumors of comparable sizes. Spermatogenesis was suppressed most among the 3 groups of thyroid-treated mice with granulosa-cell tumors, in 1 untreated mouse with a 3.2 gm. tumor, and in the estradiol benzoate-treated animals.

Three groups of experiments were designed to determine the effect of thyroid on the testicular atrophy of tumor-bearing mice. The first group received 0.2 percent thyroid daily, the second group estradiol benzoate, and the third group thyroid and estradiol benzoate. The testes of 5 male mice that received thyroid for 4½ months averaged 214 mg. (range 154–248 mg.). The seminal vesicle averaged 187 mg. (ranges 13–221 mg.). The average testicular weight of 7 untreated adult male mice was 226 mg. ($P > 0.1$). Thus thyroid acting by itself had no effect on the testicular weight. Six male mice that received 16.6 μg. of estradiol benzoate for 2½ months, followed by 33.2 μg. of estradiol benzoate for 2 months, had testicular weights averaging 217 mg. (range 186–234) and seminal-vesicle weights averaging 66 mg. (range 60–80). The testicular weights of animals that received thyroid did not differ significantly from those that received estradiol benzoate. The seminal-vesicle weights of the 2 groups differed ($P < 0.01$). Six male mice that received thyroid and estradiol benzoate in the dosages given had testes averaging 205 mg. (range 162–226) and seminal-vesicle weights averaging 91 mg. (range 63–122 mg.). The testicular and seminal-vesicle weights of estradiol benzoate-treated animals did not differ significantly from those of mice treated with both estradiol benzoate and thyroid. These experiments do not demonstrate an enhancing effect of thyroid on estrogen; thus the mode of action of thyroid in tumor-bearing animals is probably a direct one on the tumor.

Gonadotropins

The administration of FSH to female mice bearing tumor transplants from T4 and T5 donor animals had no significant effect on the percentage of takes, latent period, growth period, or hormone production of the granulosa-cell tumor (table 5).

Male T4 mice that received FSH had a longer growth period and a greater average testicular weight than their controls. The greater testicular weight of the FSH-treated mice may represent a direct stimulatory effect of FSH on the testes rather than an indirect effect mediated through a decrease in estrogen production of the tumor. FSH had no effect on the percentage of takes or the latent period (table 6).

A combination of FSH, LH, and LTH had no effect on the percentage of takes, latent period, growth period, or uterine weight of female T5 mice. The average seminal-vesicle weight of T5 male mice that received a combination of FSH, LH, and LTH was significantly less than the seminal-

TABLE 5.—Effect of gonadotropins in female mice on percentage of tumor takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean uterine weight (*mg.*)	Mean tumor weight (*gm.*)
FSH	15B T4	6	100	1	4	183	1.6
Control	15B T4	5	80	1	3	250	1.9
FSH	205 T5	7	86	2	3	125	1.1
Control	205 T5	7	100	2	2⅓	114	2.2
FSH	8 T4	6	67	2	3	132	1.7
Control	8 T4	5	60	3	3	190	1.2
FSH, LH, LTH	8 T4	5	100	2½	4	154	1.5

TABLE 6.—Effect of gonadotropins in male mice on percentage of tumor takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean testes weight (*mg.*)	Seminal vesicles mean weight (*mg.*)	Mean tumor weight (*gm.*)
FSH	8 T4	6	100	2⅓	1½*	158†	53	1.3
Control	8 T4	7	100	2	1	136	98	0.9
FSH, LH, LTH	8 T4	6	100	2	1	118	35†	0.9
FSH and castrate male 5 days after transplant	8 T4	6	67	4½†	3	—	21	0.6
FSH and castrate male 5 days after transplant	205 T5	7	86	2	1½	—	11	1.6
Control	205 T5	12	84	1½	1½	160	27	3.3
FSH, transplant 2 weeks after castration	15B T4	5	80	1	2	—	7	2.0
Control	15B T4	6	100	4	3	81	14	2.0

*$P<0.05$.
†$P<0.01$.

vesicle weight of control T5 mice. The seminal-vesicle atrophy in mice that received this combination was probably greater due to increased estrogen production of the tumor.

One group of T5 mice that was orchiectomized and given FSH 5 days after transplantation had an increase in latent period, whereas T6 mice similarly treated had latent periods similar to that of control mice. Neither group of animals differed in percentage of tumor takes nor growth period from their specific controls. T5 mice that received FSH and had tumors transplanted 2 weeks after orchiectomy had percent takes and latent periods like their control mice (*see* table 6).

Castration

None of the T5 or T6 mice that were gonadectomized had significant differences in percent takes, latent period, growth period, or uterine weight from those of control mice (tables 7 and 8).

TABLE 7.—Effect of castration in male mice on percentage of tumor takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Seminal vesicles mean weight (*gm.*)	Mean tumor weight (*gm.*)
Castration 3 days before transplant	3 T4	7	100	2	2	8.3	2.4
Control	3 T4	8	88	2	2	—	1.2
Castration 2 weeks before transplant	15B T4	5	100	¾	2½	8.1	2.2
Control	15B T4	6	100	1	3	—	2.0
Castration 2 weeks before transplant	1 T4	4	100	2	3	8.2	4.2
Control	1 T4	7	100	1½	2½	—	4.2
Castration 2 weeks after transplant	1 T4	7	86	2	4	11	3.0
Castration 4 weeks after transplant	205 T5	6	100	2	2	9.0	1.7
Control	205 T5	12	84	1½	1½	—	3.3

Steroid Hormone Treatment

Tumors in male T5 mice with a pellet of testosterone implanted each month had an increased growth period. The percentage of takes and latent period were not influenced (table 9).

TABLE 8.—Effect of castration in female mice on percentage of takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean uterine weight (*mg.*)	Mean tumor weight (*gm.*)
Castration 2 weeks before transplant	205 T5	7	86	1½	2½	121	1. 6
Control	205 T5	7	100	2	2½	114	2. 2
Castration 2 weeks before transplant	1 T4	7	100	3	4	135	2. 3
Control	1 T4	7	86	3	4	119	1. 1
Castration 2 weeks after transplant	1 T4	7	100	4	4	169	1. 7

TABLE 9.—Effect of testosterone in male mice on the percentage of tumor takes, latent period of tumor development, and growth period of a transplanted granulosa-cell tumor

Treatment*	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean tumor weight (*gm.*)
TP monthly	8 T4	6	83	2½	2†	1.2
Control	8 T4	7	100	2	1	0.9
TP and castrated 2 weeks after transplant	1 T4	7	43‡	4†	6‡	1.0
Control	1 T4	7	100	1½	2½	4.2
TP and castrated 2 weeks after transplant	15B T4	4	100	1½	4	2.1
Control	15B T4	6	100	1	3	2.0
TP and castrated 2 weeks after transplant	205 T5	7	100	1⅔	1	1.7
Control	205 T5	12	84	1½	1½	3.3
TP and castrated 2 weeks before transplant	1 T4	6	100	3	3	1.9
Control	1 T4	7	100	1½	2½	4.2

*TP = testosterone pellet.
†$P \leq 0.01$.
‡$P < 0.05$.

One group of T5 male mice that were castrated and received a testosterone pellet 2 weeks after transplantation had a decrease in percent take, an increase in latent period, and an increase in growth period.

Six female mice received 3 injections of testosterone propionate at 1, 7, and 14 days of age. The growth and function of the granulosa-cell tumors transplanted into these mice, when they reached maturity, did not differ from those in control female mice (table 10).

TABLE 10.—Effect of testosterone propionate in female mice on percentage of tumor takes, latent period of tumor development, growth period, and hormone production of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean uterine weight (*mg.*)	Mean tumor weight (*gm.*)
Testosterone propionate at birth	205 T5	6	100	2	2	115	2.4
Control	205 T5	7	100	2	2½	114	2.2
TP* and castrated 2 weeks before transplant	205 T5	6	83	1†	2	83	1.5
TP and catrasted 2 weeks before transplant	15B T4	6	83	2	3	274	1.9
Control	15B T4	5	80	1	3	250	1.9
TP and castrated 2 weeks before transplant	1 T4	7	100	2	3½	180	2.9
Control	1 T4	7	86	3	4	119	1.1
TP and castrated 2 weeks after transplant	1 T4	7	86	4	4	100	1.9

*TP = testosterone pellet.
†$P < 0.01$.

Six T5 mice that were ovariectomized and given a testosterone pellet 2 weeks prior to transplantation had a shorter latent period than control female mice. No difference in growth period, percent takes, or uterine weight could be demonstrated in these mice (table 10). Androgen-treated groups from T6 did not differ from the controls.

Male mice given weekly injections of estradiol benzoate had a longer growth period than control mice (table 11). Among female mice that received estradiol benzoate, the observations were variable (table 12). Estradiol benzoate and transplantation 2 weeks after castration had no effect on the growth of the tumors in male or female mice.

TABLE 11.—Effect of estrogen and ovarian grafts in male mice on percentage of tumor takes, latent period of tumor development, and growth period of a transplanted granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean tumor weight (*gm.*)
Control	205 T5	12	84	1½	1½	3.3
Estradiol benzoate	205 T5	9	100	1⅓	2½*	1.6
Estradiol benzoate and transplant 2 weeks after castration	205 T5	10	80	1	2	1.4
Ovarian graft and castration 2 weeks after transplant	205 T5	9	100	2	2	2.0

*$P < 0.05$.

TABLE 12.—Effect of estrogen in female mice on percentage of takes, latent period of tumor development, and growth period of a transplanted ovarian granulosa-cell tumor

Treatment	Donor animal	Number of mice	Percent of takes	Mean latent period (*months*)	Mean growth period (*months*)	Mean tumor weight (*gm.*)
Estradiol benzoate	15B T4	5	100	1½	2	3.1
Control	15B T4	5	80	1	3	1.9
Estradiol benzoate	205 T5	6	33*	¾†	4½	1.6
Control	205 T5	7	100	2	2⅓	2.2
Estradiol benzoate and transplantation 2 weeks after castration	205 T5	9	100	2	2	1.9

*$P < 0.05$.
†$P < 0.01$.

Orchiectomy and subcutaneous ovarian grafts 2 weeks prior to transplantation had no effect on the percentage of takes, latent period, and growth period.

Seven male T6 mice received cortisone from the 7th to the 13th week after transplantation. The average tumor increment was 174 mm.^{2}, whereas that of 7 untreated male mice during this same time was 112 mm.^{2} This difference between cortisone-treated and untreated male mice was not significant ($P > 0.1$).

Hypophysectomy

Eight mice were hypophysectomized after their tumor transplants became palpable. A striking initial decrease in rate of tumor growth occurred in 3 male and 5 female mice. In some, the tumor area decreased. Approximately 2½ months after hypophysectomy all tumors resumed growth.

Tumor Mass and Estrogenic Activity

Estrogenic activity of the tumor was roughly proportional to the tumor mass. The seminal vesicles were significantly smaller in mice bearing tumors that weighed more than 1 gm. when compared to animals bearing tumors that weighed less than 1 gm. and in animals with tumors weighing more than 2.5 gm. when compared to animals with tumors weighing between 1.0 and 2.5 gm. ($P < 0.01$). There was no statistically significant difference in the testicular weights of these animals. The uterine weights of T5 female mice bearing tumors weighing 1 gm. or more were larger than the average uterine weight of female mice with tumors weighing less than 1 gm. ($P < 0.01$).

Other Hormones

The production of a progesterone-like substance was indicated by the vesicular appearance of uterine stromal nuclei of the ovariectomized hosts bearing the tumors. Castrated male hosts had small seminal vesicles indicating an absence of androgen (table 7).

Histological Appearance of the Tumor and Factors That Influence It

The original tumor was a follicular type. The histological appearance of the tumor in T5 and T6 generations was a massive type. Most of the granulosa cells were arranged around sinusoids, but there were a few follicular structures. Small areas of elongated or ellipsoidal cells were found frequently, which gave the tumor a sarcomatous appearance in these areas. The transition from follicular type to massive type occurred in the first transplant generation which had components of each type. The relative extent of the follicular areas, or sarcomatous areas, could not be correlated with the tumor generation. Representative sections from untreated, hypophysectomized, and thyroid-treated animals were stained with periodic acid-Schiff's reagent (PAS), and other sections with Sudan IV and hematoxylin. PAS stained only the collagen and reticulum of the paraffin-embedded sections, and there was no difference in the appearance of the tumor in these 3 groups of animals. There was no difference in the amount of sudanophilic material in the tumors of hypophysectomized and untreated animals. The amount of sudanophilic material in the tumor differed from one area to another. The sudanophilic material was much greater in the tumors of the thyroid-treated animals than in the untreated animals.

The tumor occasionally ulcerated through the skin or penetrated the peritoneum. One mouse had a lung metastasis.

DISCUSSION

The granulosa-cell tumor exhibited qualities of malignancy and dependency. The malignancy of the tumor was manifested by its transplant-

ability, its capacity to invade the skin and peritoneum, and in at least one animal by metastasis to the lung. Its partial dependency was manifested by the modifications of its growth and function by changes in the hormonal environment of the host.

Because growth of the tumor was decreased by several different changes in the endocrine environments of the host, one might assume single or multiple mechanisms of action. Elevated or modified levels of gonadotropins have been postulated to cause ovarian tumorigenesis in ovarian grafts in the spleens of castrated mice (*9–12*).

Some experiments were designed to study whether the administration of exogenous FSH or a combination of FSH, LH, and LTH would change the biological characteristics of the tumor. Other experimental groups, which included hypophysectomy and gonadectomy, changed the endogenous level of gonadotropins. Administration of exogenous gonadotropin either singly as FSH or in combination as FSH, LH, and LTH had either inhibitory effects or no effect on tumor growth (tables 5 and 6). A combination of FSH, LH, and LTH caused increased seminal-vesicle atrophy, an effect not produced by FSH alone. Administration of exogenous LH and LTH, in addition to FSH, caused an increase in the estrogenic activity of the tumor. An increase in the level of endogenous gonadotropin produced by either ovariectomy or orchiectomy had no effect on the growth of the tumor. A decrease in endogenous gonadotropin induced by hypophysectomy was considered an initial inhibition of tumor growth. In male mice this inhibition was effective immediately postoperatively, while in female mice the tumor continued to grow in size during the first 2 weeks after hypophysectomy and then decreased in size from the 3d to the 8th week after hypophysectomy. Tumor growth in both male and female hosts resumed approximately 2½ months after hypophysectomy. This would suggest that inanition probably was not a factor in the inhibition of tumor growth since the weight of the animals showed a steady decline. The growth of the tumor in hypophysectomized mice would indicate that it was able to adjust to the new hormonal environment of the host.

The latent period of tumor development was shorter in male than in female mice. The pituitary glands of male mice produce less luteinizing hormone than those of females (*13*). Thus the higher level of LH in female mice may explain the longer latent period of tumor development.

Ovarian tumorigenesis in intrasplenic ovarian grafts in castrated mice was decreased by the administration of thyroid and thiouracil (*14*). Inhibition of the growth of transplanted granulosa-cell tumors by both thyroid and thiouracil is probably not due to the effect of these substances on the metabolic rate nor on the level of thyroid-stimulating hormone (TSH) since one would not expect opposite changes in these perimeters to bring about a single result. Several studies have revealed that induced hypothyroidism and hyperthyroidism change the responsiveness of the ovary to gonadotropins. Hybrid immature female mice given daily intraperitoneal injections of 2 or 4 μg. of thyroxin showed a greater but

more variable uterine response to FSH than did animals given FSH alone. Six μg. of thyroxin administered daily reduced the uterine response of mice given FSH. Mice that received 0.3 percent thiouracil in Purina laboratory chow had a decreased uterine response to injected FSH. Among the mice not given FSH, thiouracil reduced the uterine weight: body-weight ratio and 2 μg. of thyroxin daily augmented it, whereas 4 or 6 μg. of thyroxin reduced it (*15*). Addition of small amounts of thyroprotein to the diet of immature mice resulted in an enhanced response of the seminal vesicles and coagulating gland to the administration of pregnant-mare-serum gonadotropin (*16*).

The administration of desiccated thyroid in albino rats induced pseudopregnancy manifested by prolonged diestrous intervals and large corpora lutea. Two possible mechanisms for this action were postulated: (1) The administration of desiccated thyroid stimulates the anterior lobe of the pituitary to elaborate a hormone or hormones which bring about the formation and persistence of large functional corpora lutea, or (2) the heightened metabolism resulting from thyroid feeding may lessen the estrogen concentration to such a degree that the function of the corpora lutea may be expressed (*17*). Propylthiouracil-treated female rats that received gonadotropins derived from postmenopausal urine showed a greater degree of luteinization than normal rats that received gonadotropins (*18*). These experiments suggest that the ovary of the rat responds to hyperthyroidism and hypothyroidism by a greater degree of luteinization which may result either from an elevated LH level or greater ovarian sensitivity to a normal LH level. If a similar condition is true in mice and the granulosa-cell tumor responds in the same way as normal ovarian tissue, a change in the level of LH or a greater responsiveness to LH may explain the inhibition of tumor growth and the increased hormone production in the thyroid- and thiouracil-treated mice. The increased hormone production of the tumor in thyroid-treated mice could be correlated with an increase in sudanophilia of the tumor.

A change in the growth and function of the tumor occurred in the tumor that developed in a male mouse 2 days of age at the time of transplantation. Tumor grafts made from the tumor that developed had a decreased latent period and an increased hormone production without significant change in structure of the tumor.

Some of the changes in endocrine environment modified either the growth or the function of the tumor. Other changes caused modification in both the growth and the function. The fact that the growth and function of the tumor could be modified independently of each other suggests that these 2 qualities of the tumor are partially or completely unrelated to each other. When the growth and function were modified concomitantly, the growth was inhibited and the function augmented. The direction of this change would suggest that changes in the endocrine environment of the host partially restored the responsiveness of the tumor cells to the physiological forces which govern normal cells.

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Announcements

International Conference on the Morphological Precursors of Cancer

In June, 1961, the University of Perugia, Perugia, Italy, will celebrate the foundation of the Chair of Morbid Anatomy established in 1861-62. In honor of this occasion, three international meetings will be held in the Institute and in the Division of Cancer Research. June 25 to 30, the subject of the meeting will be "The Morphological Precursors of Cancer, in Human Beings and Animals; Hyperplasia, Metaplasia, Displasia, and Benign Tumors."

Instrument Symposium

The 10th annual instrument symposium and research equipment exhibit will be held at the National Institutes of Health, October 4–7, 1960. Sponsors are the Washington Sections of the American Association of Clinical Chemists, American Chemical Society, Instrument Society of America, Society of American Bacteriologists, and Society for Experimental Biology and Medicine. Primary topics for discussion on the scientific program include: fluorescence, infrared, activation analysis, ultracentrifuge, microscopy, and electrodes.

Fourth National Cancer Conference

The Fourth National Cancer Conference will be held at the University of Minnesota, Minneapolis, September 13–15, 1960. The theme of the Conference, "Changing Concepts Concerning Cancer," will cover three general topics: etiology, pathogenesis and spread, and therapy of malignant disease. In addition, panels of scientists will discuss the state of knowledge of the leukemias and lymphomas, and cancer of the breast, lung, gastrointestinal tract, genitourinary system, head and neck, and skin. Other panels will discuss cancer control and the role of environmental factors in the occurrence of cancer.

The Conference is sponsored jointly by the American Cancer Society and the National Cancer Institute, Public Health Service, U.S. Department of Health, Education, and Welfare. Interested scientists and physicians are invited to attend. Copies of the Conference program and registration cards may be obtained from the National Cancer Conference Coordinator, American Cancer Society, Inc., 521 West 57th Street, New York 19, N.Y.

Manuscripts Approved for JNCI, May 15 to June 15, 1960

"Reduction of transplantability of Novikoff hepatoma cells grown *in vitro* and the consequent protecting effect to the host against their malignant progenitor," by T. C. Hsu.

"Indications of involvement of the reticuloendothelial system in alterations of iron metabolism produced by tumor extracts," by Ralph F. Kampschmidt and West A. Clabaugh.

"Viruslike particles in precancerous hyperplastic mammary tissues of C3H and C3Hf mice," by Dorothy R. Pitelka, Kenneth B. DeOme, and Howard A. Bern.

"Neoplasms among A-bomb survivors in Hiroshima City," by Tomin Harada and Morihiro Ishida.

"Potential oncogenic properties of high polymers in mice," by William L. Kydd and Leo M. Sreebny.

"An electron-microscope study of spontaneous mammary carcinomas in a subline of DBA strain of mice," by Anna Goldfeder, Dorothy Gelber, and Dan H. Moore.

"Survival of patients with cancer of the stomach, Connecticut 1935–54," by Fred Ederer, Sidney J. Cutler, Henry Eisenberg, and John R. Keogh.

"Susceptibility of seven inbred strains and the F_1 hybrids to estrogen-induced testicular tumors, and occurrence of spontaneous testicular tumors in strain BALB/c mice," by Howard B. Andervont, Michael B. Shimkin, and Harry Y. Canter.

"Effect of colchicine and related substances on the morphology of peritoneal mast cells," by Jacques Padawer.

"Modified drop technique: Preparation of smears for automatic, electronic scanning," by William B. Courtney, Albert W. Hilberg, Samuel C. Ingraham II, Raymond F. Kaiser, and Mary M. Bouser.

"Immunologic response to lymphocytic choriomeningitis virus in lethally irradiated mice treated with bone marrow," by Delta E. Uphoff and Victor H. Haas.

"Mammary carcinogenesis by 3-methylcholanthrene. II. Inhibitory effect of pregnancy and lactation on tumor induction," by Thomas L. Dao, Fred G. Bock, and Mary J. Greiner.

"Occurrence of plasma-cell neoplasms in BALB/c mice injected intraperitoneally with paraffin-oil adjuvant heat-killed staphylococcus mixtures," by Michael Potter and Charlotte L. Robertson.

"Mammary tumors and mammary-gland development in hybrid mice treated with stilbestrol for varying periods," by Flavia L. Richardson and Georgia Hall.

"Relationship between chromosome ploidy and radiosensitivity in selected tumor sublines of common origin," by László Révész and Ulla Norman.

"Specific homograft tolerance induced by successive matings and implications concerning choriocarcinoma," by Richmond T. Prehn.

"Histones of cancer cells. I. Staining with ammoniacal silver," by Maurice M. Black and Francis D. Speer.

"Initial effect of community-wide cytological screening on the clinical stage of cervical cancer detected in an entire community; results in the Memphis-Shelby County, Tennessee, study," by Raymond F. Kaiser, Cyrus C. Erickson, Bennett E. Everett, Jr., Alexander G. Gilliam, Lloyd M. Graves, Mary Walton, and Douglas H. Sprunt.

"Formation of ketopentoses, beginning with ribose-5-phosphate, in the serum of patients with cancer," by Alberto Cazorla and Daniel Loayza.

"Note on the catalase activity of several mammalian cell strains after long cultivation *in vitro*," by Ellison V. Peppers, Benton B. Westfall, Harold A. Kerr, and Wilton R. Earle.

NCI Monographs

The first two monographs in the new monograph series are now available from the Superintendent of Documents, Government Printing Office, Washington 25, D.C.

Monograph No. 1:

"Estrogen-Induced Tumors of the Kidney in the Syrian Hamster," by Hadley Kirkman. Price $1.25.

Monograph No. 2:

"Symposium on Some Problems of Normal and Abnormal Differentiation and Development," edited by Nathan Kaliss. Price $1.25.

NCI Monographs 3 and 4, entitled "Conference on Experimental Clinical Cancer Chemotherapy," and "Symposium on Phenomena of the Tumor Viruses," will be published in mid-August and September 1960.

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